IARC MONOGRAPHS

GENTIAN VIOLET, LEUCOGENTIAN VIOLET, MALACHITE GREEN, LEUCOMALACHITE GREEN, AND CI DIRECT BLUE 218

VOLUME 129

IARC MONOGRAPHS ON THE IDENTIFICATION OF CARCINOGENIC HAZARDS TO HUMANS

International Agency for Research on Cancer

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This publication represents the views and expert opinions of an IARC Working Group on the Identification of Carcinogenic Hazards to Humans, which met remotely, 22 February to 5 March 2021

LYON, FRANCE - 2022

IARC MONOGRAPHS ON THE IDENTIFICATION OF CARCINOGENIC HAZARDS TO HUMANS

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In 1969, the International Agency for Research on Cancer (IARC) initiated a programme on the evaluation of the carcinogenic hazard of chemicals to humans, involving the production of critically evaluated monographs on individual chemicals. The programme was subsequently expanded to include evaluations of carcinogenic hazards associated with exposures to complex mixtures, lifestyle factors and biological and physical agents, as well as those in specific occupations. The objective of the programme is to elaborate and publish in the form of monographs critical reviews of data on carcinogenicity for agents to which humans are known to be exposed and on specific exposure situations; to evaluate these data in terms of cancer hazard to humans with the help of international working groups of experts in carcinogenesis and related fields; and to identify gaps in evidence. The lists of IARC evaluations are regularly updated and are available on the internet at https://monographs.iarc.who.int/.

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NOTE TO THE READER

The evaluations of carcinogenic hazard in the *IARC Monographs on the Identification of Carcinogenic Hazards to Humans* series are made by international working groups of independent scientists. The *IARC Monographs* classifications do not indicate the level of risk associated with a given level or circumstance of exposure. The *IARC Monographs* do not make recommendations for regulation or legislation.

Anyone who is aware of published data that may alter the evaluation of the carcinogenic hazard of an agent to humans is encouraged to make this information available to the *IARC Monographs* programme, International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France, or via email at imo@iarc.fr, in order that the agent may be considered for re-evaluation by a future Working Group.

Although every effort is made to prepare the monographs as accurately as possible, mistakes may occur. Readers are requested to communicate any errors to the *IARC Monographs* programme. Corrigenda are published online on the relevant webpage for the volume concerned (IARC Publications: https://publications.iarc.fr/).

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PREAMBLE

The Preamble to the *IARC Monographs* describes the objective and scope of the programme, general principles and procedures, and scientific review and evaluations. The *IARC Monographs* embody principles of scientific rigour, impartial evaluation, transparency, and consistency. The Preamble should be consulted when reading a *Monograph* or a summary of a *Monograph*'s evaluations. Separate Instructions for Authors describe the operational procedures for the preparation and publication of a volume of the *Monographs*.

A. GENERAL PRINCIPLES AND PROCEDURES

1. Background

Soon after the International Agency for Research on Cancer (IARC) was established in 1965, it started to receive frequent requests for advice on the carcinogenicity of chemicals, including requests for lists of established and suspected human carcinogens. In 1970, an IARC Advisory Committee on Environmental Carcinogenesis recommended "that a compendium on carcinogenic chemicals be prepared by experts. The biological activity and evaluation of practical importance to public health should be referenced and documented." The next year, the IARC Governing Council adopted a resolution that IARC should prepare "monographs on the evaluation of carcinogenic risk of chemicals to man", which became the initial title of the series.

In succeeding years, the scope of the programme broadened as *Monographs* were developed for complex mixtures, occupational exposures, physical agents, biological organisms, pharmaceuticals, and other exposures. In 1988, "of chemicals" was dropped from the title, and in 2019, "evaluation of carcinogenic risks" became "identification of carcinogenic hazards", in line with the objective of the programme.

Identifying the causes of human cancer is the first step in cancer prevention. The identification of a cancer hazard may have broad and profound implications. National and international authorities and organizations can and do use information on causes of cancer in support of actions to reduce exposure to carcinogens in the workplace, in the environment, and elsewhere. Cancer prevention is needed as much today as it was when IARC was established, because the global burden of cancer is high and continues to increase as a result of population growth and ageing and upward trends in some exposures, especially in low- and middle-income countries (https://publications.iarc.fr/Non-Series-Publications/World-Cancer-Reports).

IARC's process for developing *Monographs*, which has evolved over several decades, involves

the engagement of international, interdisciplinary Working Groups of expert scientists, the transparent synthesis of different streams of evidence (exposure characterization, cancer in humans, cancer in experimental animals, and mechanisms of carcinogenesis), and the integration of these streams of evidence into an overall evaluation and classification according to criteria developed and refined by IARC. Since the *Monographs* programme was established, the understanding of carcinogenesis has greatly deepened. Scientific advances are incorporated into the evaluation methodology. In particular, strong mechanistic evidence has had an increasing role in the overall evaluations since 1991.

The Preamble is primarily a statement of the general principles and procedures used in developing a *Monograph*, to promote transparency and consistency across *Monographs* evaluations. In addition, IARC provides Instructions for Authors (https://monographs.iarc.who.int/preamble-instructions-for-authors/), which specify more detailed working procedures. IARC routinely updates these Instructions for Authors to reflect advances in methods for cancer hazard identification and accumulated experience, including input from experts.

2. Objective and scope

The objective of the programme is to prepare, with the engagement of international, interdisciplinary Working Groups of experts, scientific reviews and evaluations of evidence on the carcinogenicity of a wide range of agents.

The *Monographs* assess the strength of the available evidence that an agent can cause cancer in humans, based on three streams of evidence: on cancer in humans (see Part B, Section 2), on cancer in experimental animals (see Part B, Section 3), and on mechanistic evidence (see Part B, Section 4). In addition, the exposure to each agent is characterized (see Part B, Section 1). In this Preamble, the term "agent" refers to any chemical, physical, or biological entity or exposure circumstance (e.g. occupation as a painter) for which evidence on the carcinogenicity is evaluated.

A cancer *hazard* is an agent that is capable of causing cancer, whereas a cancer *risk* is an estimate of the probability that cancer will occur given some level of exposure to a cancer hazard. The *Monographs* assess the strength of evidence that an agent is a cancer hazard. The distinction between hazard and risk is fundamental. The *Monographs* identify cancer hazards even when risks appear to be low in some exposure scenarios. This is because the exposure may be widespread at low levels, and because exposure levels in many populations are not known or documented.

Although the *Monographs* programme has focused on hazard identification, some epidemiological studies used to identify a cancer hazard are also used to estimate an exposure–response relationship within the range of the available data. However, extrapolating exposure–response relationships beyond the available data (e.g. to lower exposures, or from experimental animals to humans) is outside the scope of *Monographs* Working Groups (IARC, 2014). In addition, the *Monographs* programme does not review quantitative risk characterizations developed by other health agencies.

The identification of a cancer hazard should trigger some action to protect public health, either directly as a result of the hazard identification or through the conduct of a risk assessment. Although such actions are outside the scope of the programme, the *Monographs* are used by national and international authorities and organizations to inform risk assessments, formulate decisions about preventive measures, motivate effective cancer control programmes, and choose among options for public health decisions. *Monographs* evaluations are only one part of the body of information on which decisions to

control exposure to carcinogens may be based. Options to prevent cancer vary from one situation to another and across geographical regions and take many factors into account, including different national priorities. Therefore, no recommendations are given in the *Monographs* with regard to regulation, legislation, or other policy approaches, which are the responsibility of individual governments or organizations. The *Monographs* programme also does not make research recommendations. However, it is important to note that *Monographs* contribute significantly to the science of carcinogenesis by synthesizing and integrating streams of evidence about carcinogenicity and pointing to critical gaps in knowledge.

3. Selection of agents for review

Since 1984, about every five years IARC convenes an international, interdisciplinary Advisory Group to recommend agents for review by the *Monographs* programme. IARC selects Advisory Group members who are knowledgeable about current research on carcinogens and public health priorities. Before an Advisory Group meets, IARC solicits nominations of agents from scientists and government agencies worldwide. Since 2003, IARC also invites nominations from the public. IARC charges each Advisory Group with reviewing nominations, evaluating exposure and hazard potential, and preparing a report that documents the Advisory Group's process for these activities and its rationale for the recommendations.

For each new volume of the *Monographs*, IARC selects the agents for review from those recommended by the most recent Advisory Group, considering the availability of pertinent research studies and current public health priorities. On occasion, IARC may select other agents if there is a need to rapidly evaluate an emerging carcinogenic hazard or an urgent need to re-evaluate a previous classification. All evaluations consider the full body of available evidence, not just information published after a previous review.

A *Monograph* may review:

(a) An agent not reviewed in a previous *Monograph*, if there is potential human exposure and there is evidence for assessing its carcinogenicity. A group of related agents (e.g. metal compounds) may be reviewed together if there is evidence for assessing carcinogenicity for one or more members of the group.

(b) An agent reviewed in a previous *Monograph*, if there is new evidence of cancer in humans or in experimental animals, or mechanistic evidence to warrant re-evaluation of the classification. In the interests of efficiency, the literature searches may build on previous comprehensive searches.

(c) An agent that has been established to be carcinogenic to humans and has been reviewed in a previous *Monograph*, if there is new evidence of cancer in humans that indicates new tumour sites where there might be a causal association. In the interests of efficiency, the review may focus on these new tumour sites.

4. The Working Group and other meeting participants

Five categories of participants can be present at *Monographs* meetings:

(i) *Working Group* members are responsible for all scientific reviews and evaluations developed in the volume of the *Monographs*. The Working Group is interdisciplinary and comprises subgroups of experts in the fields of (a) exposure characterization, (b) cancer in humans, (c) cancer in experimental animals, and (d) mechanistic evidence. IARC selects Working Group members on the basis of

expertise related to the subject matter and relevant methodologies, and absence of conflicts of interest. Consideration is also given to diversity in scientific approaches and views, as well as demographic composition. Working Group members generally have published research related to the exposure or carcinogenicity of the agents being reviewed, and IARC uses literature searches to identify most experts. Since 2006, IARC also has encouraged public nominations through its Call for Experts. IARC's reliance on experts with knowledge of the subject matter and/or expertise in methodological assessment is confirmed by decades of experience documenting that there is value in specialized expertise and that the overwhelming majority of Working Group members are committed to the objective evaluation of scientific evidence and not to the narrow advancement of their own research results or a pre-determined outcome (Wild & Cogliano, 2011). Working Group members are expected to serve the public health mission of IARC, and should refrain from consulting and other activities for financial gain that are related to the agents under review, or the use of inside information from the meeting, until the full volume of the *Monographs* is published.

IARC identifies, from among Working Group members, individuals to serve as Meeting Chair and Subgroup Chairs. At the opening of the meeting, the Working Group is asked to endorse the selection of the Meeting Chair, with the opportunity to propose alternatives. The Meeting Chair and Subgroup Chairs take a leading role at all stages of the review process (see Part A, Section 7), promote open scientific discussions that involve all Working Group members in accordance with normal committee procedures, and ensure adherence to the Preamble.

(ii) *Invited Specialists* are experts who have critical knowledge and experience but who also have a conflict of interest that warrants exclusion from developing or influencing the evaluations of carcinogenicity. Invited Specialists do not draft any section of the *Monograph* that pertains to the description or interpretation of cancer data, and they do not participate in the evaluations. These experts are invited in limited numbers when necessary to assist the Working Group by contributing their unique knowledge and experience to the discussions.

(iii) *Representatives of national and international health agencies* may attend because their agencies are interested in the subject of the meeting. They do not draft any section of the *Monograph* or participate in the evaluations.

(iv) *Observers* with relevant scientific credentials may be admitted in limited numbers. Attention is given to the balance of Observers from constituencies with differing perspectives. Observers are invited to observe the meeting and should not attempt to influence it, and they agree to respect the Guidelines for Observers at *IARC Monographs* meetings. Observers do not draft any section of the *Monograph* or participate in the evaluations.

(v) The *IARC Secretariat* consists of scientists who are designated by IARC and who have relevant expertise. The IARC Secretariat coordinates and facilitates all aspects of the evaluation and ensures adherence to the Preamble throughout development of the scientific reviews and classifications (see Part A, Sections 5 and 6). The IARC Secretariat organizes and announces the meeting, identifies and recruits the Working Group members, and assesses the declared interests of all meeting participants. The IARC Secretariat supports the activities of the Working Group (see Part A, Section 7) by

Table 1 Roles of participants at *IARC Monographs* meetings

Category of participant	Role			
	Prepare text, tables, and analyses	Participate in discussions	Participate in evaluations	Eligible to serve as Chair
Working Group members	✓	✓	✓	✓
Invited Specialists	✓[a]	✓		
Representatives of health agencies		✓[b]		
Observers		✓[b]		
IARC Secretariat	✓[c]	✓	✓[d]	

[a] Only for the section on exposure characterization.
[b] Only at times designated by the Meeting Chair and Subgroup Chairs.
[c] When needed or requested by the Meeting Chair and Subgroup Chairs.
[d] Only for clarifying or interpreting the Preamble.

searching the literature and performing title and abstract screening, organizing conference calls to coordinate the development of pre-meeting drafts and discuss cross-cutting issues, and reviewing drafts before and during the meeting. Members of the IARC Secretariat serve as meeting rapporteurs, assist the Meeting Chair and Subgroup Chairs in facilitating all discussions, and may draft text or tables when designated by the Meeting Chair and Subgroup Chairs. Their participation in the evaluations is restricted to the role of clarifying or interpreting the Preamble.

All participants are listed, with their principal affiliations, in the front matter of the published volume of the *Monographs*. Working Group members and Invited Specialists serve as individual scientists and not as representatives of any organization, government, or industry (Cogliano et al., 2004).

The roles of the meeting participants are summarized in Table 1.

5. Working procedures

A separate Working Group is responsible for developing each volume of the *Monographs*. A volume contains one or more *Monographs*, which can cover either a single agent or several related agents. Approximately one year before the meeting of a Working Group, a preliminary list of agents to be reviewed, together with a Call for Data and a Call for Experts, is announced on the *Monographs* programme website (https://monographs.iarc.who.int/).

Before a meeting invitation is extended, each potential participant, including the IARC Secretariat, completes the WHO Declaration of Interests form to report financial interests, employment and consulting (including remuneration for serving as an expert witness), individual and institutional research support, and non-financial interests such as public statements and positions related to the subject of the meeting. IARC assesses the declared interests to determine whether there is a conflict that warrants any limitation on participation (see Table 2).

Approximately two months before a *Monographs* meeting, IARC publishes the names and affiliations of all meeting participants together with a summary of declared interests, in the interests of transparency and to provide an opportunity for undeclared conflicts of interest to be brought to IARC's attention. It is not acceptable for Observers or third parties to contact other participants before a meeting or to lobby them at any time. Meeting participants are asked to report all such contacts to IARC (Cogliano et al., 2005).

Table 2 Public engagement during *Monographs* development

Approximate timeframe	Engagement
Every 5 years	IARC convenes an Advisory Group to recommend high-priority agents for future review
~1 year before a *Monographs* meeting	IARC selects agents for review in a new volume of the *Monographs* IARC posts on its website: Preliminary List of Agents to be reviewed Call for Data and Call for Experts Request for Observer Status WHO Declaration of Interests form
~8 months before a *Monographs* meeting	Call for Experts closes
~4 months before a *Monographs* meeting	Request for Observer Status closes
~2 months before a *Monographs* meeting	IARC posts the names of all meeting participants together with a summary of declared interests, and a statement discouraging contact of the Working Group by interested parties
~1 month before a *Monographs* meeting	Call for Data closes
~2–4 weeks after a *Monographs* meeting	IARC publishes a summary of evaluations and key supporting evidence
~9 months after a *Monographs* meeting	IARC Secretariat publishes the verified and edited master copy of plenary drafts as a *Monographs* volume

The Working Group meets at IARC for approximately eight days to discuss and finalize the scientific review and to develop summaries and evaluations. At the opening of the meeting, all participants update their Declaration of Interests forms, which are then reviewed by IARC. Declared interests related to the subject of the meeting are disclosed to the meeting participants during the meeting and in the published volume (Cogliano et al., 2004). The objectives of the meeting are peer review and consensus. During the first part of the meeting, subgroup sessions (covering exposure characterization, cancer in humans, cancer in experimental animals, and mechanistic evidence) review the pre-meeting drafts, develop a joint subgroup draft, and draft subgroup summaries. During the last part of the meeting, the Working Group meets in plenary session to review the subgroup drafts and summaries and to develop the consensus evaluations. As a result, the entire volume is the joint product of the Working Group, and there are no individually authored sections. After the meeting, the master copy is verified by the IARC Secretariat and is then edited and prepared for publication. The aim is to publish the volume within approximately nine months of the Working Group meeting. A summary of the evaluations and key supporting evidence is prepared for publication in a scientific journal or is made available on the *Monographs* programme website soon after the meeting.

In the interests of transparency, IARC engages with the public throughout the process, as summarized in Table 2.

6. Overview of the scientific review and evaluation process

The Working Group considers all pertinent epidemiological studies, cancer bioassays in experimental animals, and mechanistic evidence, as well as pertinent information on exposure in humans. In general, for cancer in humans, cancer in experimental animals, and mechanistic evidence, only studies that have been published or accepted for publication in the openly available scientific literature are reviewed. Under some circumstances, materials

that are publicly available and whose content is final may be reviewed if there is sufficient information to permit an evaluation of the quality of the methods and results of the studies (see Step 1, below). Such materials may include reports and databases publicly available from government agencies, as well as doctoral theses. The reliance on published and publicly available studies promotes transparency and protects against citation of premature information.

The principles of systematic review are applied to the identification, screening, synthesis, and evaluation of the evidence related to cancer in humans, cancer in experimental animals, and mechanistic evidence (as described in Part B, Sections 2–4 and as detailed in the Instructions for Authors). Each *Monograph* specifies or references information on the conduct of the literature searches, including search terms and inclusion/exclusion criteria that were used for each stream of evidence.

In brief, the steps of the review process are as follows:

Step 1. Comprehensive and transparent identification of the relevant information: The IARC Secretariat identifies relevant studies through initial comprehensive searches of literature contained in authoritative biomedical databases (e.g. PubMed, PubChem) and through a Call for Data. These literature searches, designed in consultation with a librarian and other technical experts, address whether the agent causes cancer in humans, causes cancer in experimental systems, and/or exhibits key characteristics of established human carcinogens (in humans or in experimental systems). The Working Group provides input and advice to IARC to refine the search strategies, and identifies literature through other searches (e.g. from reference lists of past *Monographs*, retrieved articles, and other authoritative reviews).

For certain types of agents (e.g. regulated pesticides and pharmaceuticals), IARC also provides an opportunity to relevant regulatory authorities, and regulated parties through such authorities, to make pertinent unpublished studies publicly available by the date specified in the Call for Data. Consideration of such studies by the Working Group is dependent on the public availability of sufficient information to permit an independent evaluation of (a) whether there has been selective reporting (e.g. on outcomes, or from a larger set of conducted studies); (b) study quality (e.g. design, methodology, and reporting of results), and (c) study results.

Step 2. Screening, selection, and organization of the studies: The IARC Secretariat screens the retrieved literature for inclusion based on title and abstract review, according to pre-defined exclusion criteria. For instance, studies may be excluded if they were not about the agent (or a metabolite of the agent), or if they reported no original data on epidemiological or toxicological end-points (e.g. review articles). The Working Group reviews the title and abstract screening done by IARC, and performs full-text review. Any reasons for exclusion are recorded, and included studies are organized according to factors pertinent to the considerations described in Part B, Sections 2–4 (e.g. design, species, and end-point). Inclusion of a study does not imply acceptance of the adequacy of the study design or of the analysis and interpretation of the results.

Step 3. Evaluation of study quality: The Working Group evaluates the quality of the included studies based on the considerations (e.g. design, methodology, and reporting of results) described in Part B, Sections 2–4. Based on these considerations, the Working Group may accord greater weight to some of the included studies. Interpretation of the

results and the strengths and limitations of a study are clearly outlined in square brackets at the end of study descriptions (see Part B).

Step 4: *Report characteristics of included studies, including assessment of study quality*: Pertinent characteristics and results of included studies are reviewed and succinctly described, as detailed in Part B, Sections 1–4. Tabulation of data may facilitate this reporting. This step may be iterative with Step 3.

Step 5: *Synthesis and evaluation of strength of evidence*: The Working Group summarizes the overall strengths and limitations of the evidence from the individual streams of evidence (cancer in humans, cancer in experimental animals, and mechanistic evidence; see Part B, Section 5). The Working Group then evaluates the strength of evidence from each stream of evidence by using the transparent methods and defined descriptive terms given in Part B, Sections 6a–c. The Working Group then develops, and describes the rationale for, the consensus classification of carcinogenicity that integrates the conclusions about the strength of evidence from studies of cancer in humans, studies of cancer in experimental animals, and mechanistic evidence (see Part B, Section 6d).

7. Responsibilities of the Working Group

The Working Group is responsible for identifying and evaluating the relevant studies and developing the scientific reviews and evaluations for a volume of the *Monographs*. The IARC Secretariat supports these activities of the Working Group (see Part A, Section 4). Briefly, the Working Group's tasks in developing the evaluation are, in sequence:

(i) Before the meeting, the Working Group ascertains that all appropriate studies have been identified and selected, and assesses the methods and quality of each individual study, as outlined above (see Part A, Section 6). The Working Group members prepare pre-meeting working drafts that present accurate tabular or textual summaries of informative studies by extracting key elements of the study design and results, and highlighting notable strengths and limitations. They participate in conference calls organized by IARC to coordinate the development of working drafts and to discuss cross-cutting issues. Pre-meeting reviews of all working drafts are generally performed by two or more subgroup members who did not participate in study identification, data extraction, or study review for the draft. Each study summary is written or reviewed by someone who is not associated with the study.

(ii) At the meeting, within subgroups, the Working Group members critically review, discuss, and revise the pre-meeting drafts and adopt the revised versions as consensus subgroup drafts. Subgroup Chairs ensure that someone who is not associated with the study leads the discussion of each study summary. A proposed classification of the strength of the evidence reviewed in the subgroup using the *IARC Monographs* criteria (see Part B, Sections 6a–c) is then developed from the consensus subgroup drafts of the evidence summaries (see Part B, Section 5).

(iii) During the plenary session, each subgroup presents its drafts for scientific review and discussion to the other Working Group members, who did not participate in study identification, data extraction, or study review for the drafts. Subgroup Chairs ensure that someone who is not associated with the study leads the discussion of each study summary.

After review, discussion, and revisions as needed, the subgroup drafts are adopted as a consensus Working Group product. The summaries and classifications of the strength of the evidence, developed in the subgroup in line with the *IARC Monographs* criteria (see Part B, Sections 6a–c), are considered, revised as needed, and adopted by the full Working Group. The Meeting Chair proposes an overall evaluation using the guidance provided in Part B, Section 6d.

The Working Group strives to achieve consensus evaluations. Consensus reflects broad agreement among the Working Group, but not necessarily unanimity. The Meeting Chair may poll the Working Group to determine the diversity of scientific opinion on issues where consensus is not apparent.

Only the final product of the plenary session represents the views and expert opinions of the Working Group. The entire *Monographs* volume is the joint product of the Working Group and represents an extensive and thorough peer review of the body of evidence (individual studies, synthesis, and evaluation) by an interdisciplinary expert group. Initial working papers and subsequent revisions are not released, because they would give an incomplete and possibly misleading impression of the consensus developed by the Working Group over a full week of deliberation.

B. SCIENTIFIC REVIEW AND EVALUATION

This part of the Preamble discusses the types of evidence that are considered and summarized in each section of a *Monograph*, followed by the scientific criteria that guide the evaluations. In addition, a section of General Remarks at the front of the volume discusses the reasons the agents were scheduled for evaluation and any key issues encountered during the meeting.

1. Exposure characterization

This section identifies the agent and describes its occurrence, main uses, and production locations and volumes, where relevant. It also summarizes the prevalence, concentrations in relevant studies, and relevant routes of exposure in humans worldwide. Methods of exposure measurement and analysis are described, and methods of exposure assessment used in key epidemiological studies reviewed by the Working Group are described and evaluated.

Over the course of the *Monographs* programme, concepts of exposure and dose have evolved substantially with deepening understanding of the interactions of agents and biological systems. The concept of exposure has broadened and become more holistic, extending beyond chemical, physical, and biological agents to stressors as construed generally, including psychosocial stressors (National Research Council, 2012; National Academies of Sciences, Engineering, and Medicine, 2017). Overall, this broader conceptualization supports greater integration between exposure characterization and other sections of the *Monographs*. Concepts of absorption, distribution, metabolism, and excretion are considered in the first subsection of mechanistic evidence (see Part B, Section 4a), whereas validated biomarkers of internal exposure or metabolites that are routinely used for exposure assessment are reported on in this section (see Part B, Section 1b).

(a) Identification of the agent

The agent being evaluated is unambiguously identified. Details will vary depending on the type of agent but will generally include physical and chemical properties relevant to the agent's identification, occurrence, and biological activity.

If the material that has been tested in experimental animals or in vitro systems is different from that to which humans are exposed, these differences are noted.

For chemical agents, the Chemical Abstracts Service Registry Number is provided, as well as the latest primary name and other names in common use, including important trade names, along with available information on the composition of common mixtures or products containing the agent, and potentially toxic and/or carcinogenic impurities. Physical properties relevant to understanding the potential for human exposure and measures of exposure used in studies in humans are summarized. These might include physical state, volatility, aqueous and fat solubility, and half-life in the environment and/or in human tissues.

For biological agents, taxonomy and structure are described. Mode of replication, life-cycle, target cells, persistence, latency, and host responses, including morbidity and mortality through pathologies other than cancer, are also presented.

For foreign bodies, fibres and particles, composition, size range, relative dimensions, and accumulation, persistence, and clearance in target organs are summarized. Physical agents that are forms of radiation are described in terms of frequency spectrum and energy transmission.

Exposures may result from, or be influenced by, a diverse range of social and environmental factors, including components of diet, sleep, and physical activity patterns. In these instances, this section will include a description of the agent, its variability across human populations, and its composition or characteristics relevant to understanding its potential carcinogenic hazard to humans and to evaluating exposure assessments in epidemiological studies.

(b) Detection and analysis

Key methods of detection and quantification of the agent are presented, with an emphasis on those used most widely in surveillance, regulation, and epidemiological studies. Measurement methods for sample matrices that are deemed important sources of human exposure (e.g. air, drinking-water, food, residential dust) and for validated exposure biomarkers (e.g. the agent or its metabolites in human blood, urine, or saliva) are described. Information on detection and quantification limits is provided when it is available and is useful for interpreting studies in humans and in experimental animals. This is not an exhaustive treatise but is meant to help readers understand the strengths and limitations of the available exposure data and of the epidemiological studies that rely on these measurements.

(c) Production and use

Historical and geographical patterns and trends in production and use are included when they are available, to help readers understand the contexts in which exposures may occur, both within key epidemiological studies reviewed by the Working Group and in human populations generally. Industries that produce, use, or dispose of the agent are described, including their global distribution, when available. National or international listing as a high-production-volume chemical or similar classification may be included. Production processes with significant potential for occupational exposure or environmental pollution are indicated. Trends in global production volumes, technologies, and other data relevant to understanding exposure potential are summarized. Minor or historical uses with significant exposure potential or with particular relevance to key epidemiological studies are included. Particular effort may be directed towards finding data on production in low- and middle-income countries, where rapid

economic development may lead to higher exposures than those in high-income countries.

(d) Exposure

A concise overview of quantitative information on sources, prevalence, and levels of exposure in humans is provided. Representative data from research studies, government reports and websites, online databases, and other citable, publicly available sources are tabulated. Data from low- and middle-income countries are sought and included to the extent feasible; information gaps for key regions are noted. Naturally occurring sources of exposure, if any, are noted. Primary exposure routes (e.g. inhalation, ingestion, skin uptake) and other considerations relevant to understanding the potential for cancer hazard from exposure to the agent are reported.

For occupational settings, information on exposure prevalence and levels (e.g. in air or human tissues) is reported by industry, occupation, region, and other characteristics (e.g. process, task) where feasible. Information on historical exposure trends, protection measures to limit exposure, and potential co-exposures to other carcinogenic agents in workplaces is provided when available.

For non-occupational settings, the occurrence of the agent is described with environmental monitoring or surveillance data. Information on exposure prevalence and levels (e.g. concentrations in human tissues) as well as exposure from and/or concentrations in food and beverages, consumer products, consumption practices, and personal microenvironments is reported by region and other relevant characteristics. Particular importance is placed on describing exposures in life stages or in states of disease or nutrition that may involve greater exposure or susceptibility.

Current exposures are of primary interest; however, information on historical exposure trends is provided when available. Historical exposures may be relevant for interpreting epidemiological studies, and when agents are persistent or have long-term effects. Information gaps for important time periods are noted. Exposure data that are not deemed to have high relevance to human exposure are generally not considered.

(e) Regulations and guidelines

Regulations or guidelines that have been established for the agent (e.g. occupational exposure limits, maximum permitted levels in foods and water, pesticide registrations) are described in brief to provide context about government efforts to limit exposure; these may be tabulated if they are informative for the interpretation of existing or historical exposure levels. Information on applicable populations, specific agents concerned, basis for regulation (e.g. human health risk, environmental considerations), and timing of implementation may be noted. National and international bans on production, use, and trade are also indicated.

This section aims to include major or illustrative regulations and may not be comprehensive, because of the complexity and range of regulatory processes worldwide. An absence of information on regulatory status should not be taken to imply that a given country or region lacks exposure to, or regulations on exposure to, the agent.

(f) Critical review of exposure assessment in key epidemiological studies

Epidemiological studies evaluate cancer hazard by comparing outcomes across differently exposed groups. Therefore, the type and quality of the exposure assessment methods used are key considerations when interpreting study findings for hazard identification. This section summarizes and critically reviews the exposure assessment methods used in the individual epidemiological studies that contribute data relevant to the *Monographs* evaluation.

Although there is no standard set of criteria for evaluating the quality of exposure assessment methods across all possible agents, some concepts are universally relevant. Regardless of the agent, all exposures have two principal dimensions: intensity (sometimes defined as concentration or dose) and time. Time considerations include duration (time from first to last exposure), pattern or frequency (whether continuous or intermittent), and windows of susceptibility. This section considers how each of the key epidemiological studies characterizes these dimensions. Interpretation of exposure information may also be informed by consideration of mechanistic evidence (e.g. as described in Part B, Section 4a), including the processes of absorption, distribution, metabolism, and excretion.

Exposure intensity and time in epidemiological studies can be characterized by using environmental or biological monitoring data, records from workplaces or other sources, expert assessments, modelled exposures, job-exposure matrices, and subject or proxy reports via questionnaires or interviews. Investigators use these data sources and methods individually or in combination to assign levels or values of an exposure metric (which may be quantitative, semi-quantitative, or qualitative) to members of the population under study.

In collaboration with the Working Group members reviewing human studies (of cancer and of mechanisms), key epidemiological studies are identified. For each selected study, the exposure assessment approach, along with its strengths and limitations, is summarized using text and tables. Working Group members identify concerns about exposure assessment methods and their impacts on overall quality for each study reviewed (see Part B, Sections 2d and 4d). In situations where the information provided in the study is inadequate to properly consider the exposure assessment, this is indicated. When adequate information is available, the likely direction of bias due to error in exposure measurement, including misclassification (overestimated effects, underestimated effects, or unknown) is discussed.

2. Studies of cancer in humans

This section includes all pertinent epidemiological studies (see Part B, Section 2b) that include cancer as an outcome. These studies encompass certain types of biomarker studies, for example, studies with biomarkers as exposure metrics (see Part B, Section 2) or those evaluating histological or tumour subtypes and molecular signatures in tumours consistent with a given exposure (Alexandrov et al., 2016). Studies that evaluate early biological effect biomarkers are reviewed in Part B, Section 4.

(a) Types of study considered

Several types of epidemiological studies contribute to the assessment of carcinogenicity in humans; they typically include cohort studies (including variants such as case–cohort and nested case–control studies), case–control studies, ecological studies, and intervention studies. Rarely, results from randomized trials may be available. Exceptionally, case reports and case series of cancer in humans may also be reviewed. In addition to these designs, innovations in epidemiology allow for many other variants that may be considered in any given *Monographs* evaluation.

Cohort and case–control studies typically have the capacity to relate individual exposures under study to the occurrence of cancer in individuals, and provide an estimate of effect (such as relative risk) as the main measure of association. Well-conducted cohort and case–control studies provide most of the evidence of cancer in humans evaluated by Working Groups. Intervention studies are much less common, but when available can provide strong evidence for making causal inferences.

In ecological studies, the units of investigation are usually whole populations (e.g. in particular geographical areas or at particular times), and cancer frequency is related to a summary measure of the exposure in the population under study. In ecological studies, data on individual exposure and outcome are not available, which renders this type of study more prone to confounding and exposure misclassification. In some circumstances, however, ecological studies may be informative, especially when the unit of exposure is most accurately measured at the population level (see, for example, the *Monograph* on arsenic in drinking-water; IARC, 2004).

Exceptionally, case reports and case series may provide compelling evidence about the carcinogenicity of an agent. In fact, many of the early discoveries of occupational cancer hazards came about because of observations by workers and their clinicians, who noted a high frequency of cancer in workers who share a common occupation or exposure. Such observations may be the starting point for more structured investigations, but in exceptional circumstances, when the risk is high enough, the case series may in itself provide compelling evidence. This would be especially warranted in situations where the exposure circumstance is fairly unusual, as it was in the example of plants containing aristolochic acid (IARC, 2012a).

The uncertainties that surround the interpretation of case reports, case series, and ecological studies typically make them inadequate, except in rare instances as described above, to form the sole basis for inferring a causal relationship. However, when considered together with cohort and case–control studies, these types of study may support the judgement that a causal relationship exists.

Epidemiological studies of benign neoplasms, pre-neoplastic lesions, malignant precursors, and other end-points are also reviewed when they relate to the agents reviewed. On occasion they can strengthen inferences drawn from studies of cancer itself. For example, benign brain tumours may share common risk factors with those that are malignant, and benign neoplasms (or those of uncertain behaviour) may be part of the causal path to malignancies (e.g. myelodysplastic syndromes, which may progress to acute myeloid leukaemia).

(b) Identification of eligible studies of cancer in humans

Relevant studies of cancer in humans are identified by using systematic review principles as described in Part A, further elaborated in the Instructions for Authors, and as detailed below. Eligible studies include all studies in humans of exposure to the agent of interest with cancer as an outcome. Multiple publications on the same study population are identified so that the number of independent studies is accurately represented. Multiple publications may result, for example, from successive follow-ups of a single cohort, from analyses focused on different aspects of an exposure–disease association, or from inclusion of overlapping populations. Usually in such situations, only the most recent, most comprehensive, or most informative report is reviewed in detail.

(c) Assessment of study quality and informativeness

Epidemiological studies are potentially susceptible to several different sources of error, summarized briefly below. Qualities of individual studies that address these issues are also described below.

Study quality is assessed as part of the structured expert review process undertaken by the Working Group. A key aspect of quality assessment is consideration of the possible roles of chance and bias in the interpretation of epidemiological studies. Chance, which is also called

random variation, can produce misleading study results. This variability in study results is strongly influenced by the sample size: smaller studies are more likely than larger studies to have effect estimates that are imprecise. Confidence intervals around a study's point estimate of effect are used routinely to indicate the range of values of the estimate that could easily be produced by chance alone.

Bias is the effect of factors in study design or conduct that lead an association to erroneously appear stronger or weaker than the association that really exists between the agent and the disease. Biases that require consideration are varied but are usually categorized as selection bias, information bias (e.g. error in measurement of exposure and diseases), and confounding (or confounding bias), (Rothman et al., 2008). Selection bias in an epidemiological study occurs when inclusion of participants from the eligible population or their follow-up in the study is influenced by their exposure or their outcome (usually disease occurrence). Under these conditions, the measure of association found in the study will not accurately reflect the association that would otherwise have been found in the eligible population (Hernán et al., 2004). Information bias results from inaccuracy in exposure or outcome measurement. Both can cause an association between hypothesized cause and effect to appear stronger or weaker than it really is. Confounding is a mixing of extraneous effects with the effects of interest (Rothman et al., 2008). An association between the purported causal factor and another factor that is associated with an increase or decrease in incidence of disease can lead to a spurious association or absence of a real association of the presumed causal factor with the disease. When either of these occurs, confounding is present.

In assessing study quality, the Working Group consistently considers the following aspects:

- **Study description:** Clarity in describing the study design and its implementation, and the completeness of reporting of all other key information about the study and its results.
- **Study population:** Whether the study population was appropriate for evaluating the association between the agent and cancer. Whether the study was designed and carried out to minimize selection bias. Cancer cases in the study population must have been identified in a way that was independent of the exposure of interest, and exposure assessed in a way that was not related to disease (outcome) status. In these respects, completeness of recruitment into the study from the population of interest and completeness of follow-up for the outcome are essential measures.
- **Outcome measurement:** The appropriateness of the cancer outcome measure (e.g. mortality vs incidence) for the agent and cancer type under consideration, outcome ascertainment methodology, and the extent to which outcome misclassification may have led to bias in the measure(s) of association.
- **Exposure measurement:** The adequacy of the methods used to assess exposure to the agent, and the likelihood (and direction) of bias in the measure(s) of association due to error in exposure measurement, including misclassification (as described in Part B, Section 1f).
- **Assessment of potential confounding:** To what extent the authors took into account in the study design and analysis other variables (including co-exposures, as described in Part B, Section 1d) that can influence the risk of disease and may have been related to the exposure of interest. Important sources of potential confounding by such variables should have been addressed either in the design of the study, such as by matching or restriction, or in the analysis, by statistical adjustment. In some instances, where direct information on confounders is unavailable,

use of indirect methods to evaluate the potential impact of confounding on exposure–disease associations is appropriate (e.g. Axelson & Steenland, 1988; Richardson et al., 2014).

- **Other potential sources of bias:** Each epidemiological study is unique in its study population, its design, its data collection, and, consequently, its potential biases. All possible sources of bias are considered for their possible impact on the results. The possibility of reporting bias (i.e. selective reporting of some results and the suppression of others) should be explored.
- **Statistical methodology:** Adequacy of the statistical methods used and their ability to obtain unbiased estimates of exposure–outcome associations, confidence intervals, and test statistics for the significance of measures of association. Appropriateness of methods used to investigate confounding, including adjusting for matching when necessary and avoiding treatment of probable mediating variables as confounders. Detailed analyses of cancer risks in relation to summary measures of exposure such as cumulative exposure, or temporal variables such as age at first exposure or time since first exposure, are reviewed and summarized when available.

For the sake of economy and simplicity, in this Preamble the list of possible sources of error is referred to with the phrase "chance, bias, and confounding", but it should be recognized that this phrase encompasses a comprehensive set of concerns pertaining to study quality.

These sources of error do not constitute and should not be used as a formal checklist of indicators of study quality. The judgement of experienced experts is critical in determining how much weight to assign to different issues in considering how all of these potential sources of error should be integrated and how to rate the potential for error related to each of these considerations.

The informativeness of a study is its ability to show a true association, if there is one, between the agent and cancer, and the lack of an association, if no association exists. Key determinants of informativeness include: having a study population of sufficient size to obtain precise estimates of effect; sufficient elapsed time from exposure to measurement of outcome for an effect, if present, to be observable; presence of an adequate exposure contrast (intensity, frequency, and/or duration); biologically relevant definitions of exposure; and relevant and well-defined time windows for exposure and outcome.

(d) Meta-analyses and pooled analyses

Independent epidemiological studies of the same agent may lead to inconsistent results that are difficult to interpret or reconcile. Combined analyses of data from multiple studies may be conducted as a means to address this ambiguity. There are two types of combined analysis. The first involves combining summary statistics such as relative risks from individual studies (meta-analysis), and the second involves a pooled analysis of the raw data from the individual studies (pooled analysis) (Greenland & O'Rourke, 2008).

The strengths of combined analyses are increased precision because of increased sample size and, in the case of pooled analyses, the opportunity to better control for potential confounders and to explore in more detail interactions and modifying effects that may explain heterogeneity among studies. A disadvantage of combined analyses is the possible lack of comparability of data from various studies, because of differences in population characteristics, subject recruitment, procedures of data collection, methods of measurement, and effects of unmeasured covariates that may differ among studies. These differences in study methods and quality can influence

results of either meta-analyses or pooled analyses. If published meta-analyses are to be considered by the Working Group, their adequacy needs to be carefully evaluated, including the methods used to identify eligible studies and the accuracy of data extracted from the individual studies.

The Working Group may conduct ad hoc meta-analyses during the course of a *Monographs* meeting, when there are sufficient studies of an exposure–outcome association to contribute to the Working Group's assessment of the association. The results of such unpublished original calculations, which would be specified in the text by presentation in square brackets, might involve updates of previously conducted analyses that incorporate the results of more recent studies, or de novo analyses.

Irrespective of the source of data for the meta-analyses and pooled analyses, the following key considerations apply: the same criteria for data quality must be applied as for individual studies; sources of heterogeneity among studies must be carefully considered; and the possibility of publication bias should be explored.

(e) Considerations in assessing the body of epidemiological evidence

The ability of the body of epidemiological evidence to inform the Working Group about the carcinogenicity of the agent is related to both the quantity and the quality of the evidence. There is no formulaic answer to the question of how many studies of cancer in humans are needed from which to draw inferences about causality, although more than a single study in a single population will almost always be needed. The number will depend on the considerations relating to evidence described below.

After the quality of individual epidemiological studies of cancer has been assessed and the informativeness of the various studies on the association between the agent and cancer has been evaluated, a judgement is made about the strength of evidence that the agent in question is carcinogenic to humans. In making its judgement, the Working Group considers several aspects of the body of evidence (e.g. Hill, 1965; Rothman et al., 2008; Vandenbroucke et al., 2016).

A strong association (e.g. a large relative risk) is more likely to indicate causality than is a weak association, because it is more difficult for confounding to falsely create a strong association. However, it is recognized that estimates of effect of small magnitude do not imply lack of causality and may have impact on public health if the disease or exposure is common. Estimates of effect of small magnitude could also contribute useful information to the assessment of causality if level of risk is commensurate with level of exposure when compared with risk estimates from populations with higher exposure (e.g. as seen in residential radon studies compared with studies of radon from uranium mining).

Associations that are consistently observed in several studies of the same design, or in studies that use different epidemiological approaches, or under different circumstances of exposure are more likely to indicate a causal relationship than are isolated observations from single studies. If there are inconsistent results among investigations, possible reasons are sought (e.g. differences in study informativeness because of latency, exposure levels, or assessment methods). Results of studies that are judged to be of high quality and informativeness are given more weight than those of studies judged to be methodologically less sound or less informative.

Temporality of the association is an essential consideration: that is, the exposure must precede the outcome.

An observation that cancer risk increases with increasing exposure is considered to be a strong indication of causality, although the absence of a graded response is not necessarily evidence against a causal relationship, and there are several reasons why the shape of the exposure–response

association may be non-monotonic (e.g. Stayner et al., 2003). The demonstration of a decline in risk after cessation of or reduction in exposure in individuals or in whole populations also supports a causal interpretation of the findings.

Confidence in a causal interpretation of the evidence from studies of cancer in humans is enhanced if it is coherent with physiological and biological knowledge, including information about exposure to the target organ, latency and timing of the exposure, and characteristics of tumour subtypes.

The Working Group considers whether there are subpopulations with increased susceptibility to cancer from the agent. For example, molecular epidemiology studies that identify associations between genetic polymorphisms and inter-individual differences in cancer susceptibility to the agent(s) being evaluated may contribute to the identification of carcinogenic hazards to humans. Such studies may be particularly informative if polymorphisms are found to be modifiers of the exposure–response association, because evaluation of polymorphisms may increase the ability to detect an effect in susceptible subpopulations.

When, in the process of evaluating the studies of cancer in humans, the Working Group identifies several high-quality, informative epidemiological studies that clearly show either no positive association or an inverse association between an exposure and a specific type of cancer, a judgement may be made that, in the aggregate, they suggest evidence of lack of carcinogenicity for that cancer type. Such a judgement requires, first, that the studies strictly meet the standards of design and analysis described above. Specifically, the possibility that bias, confounding, or misclassification of exposure or outcome could explain the observed results should be considered and ruled out with reasonable confidence. In addition, all studies that are judged to be methodologically sound should (a) be consistent with an estimate of relative effect of unity (or below unity) for any observed level of exposure, (b) when considered together, provide a combined estimate of relative risk that is at or below unity, and (c) have a narrow confidence interval. Moreover, neither any individual well-designed and well-conducted study nor the pooled results of all the studies should show any consistent tendency that the relative risk of cancer increases with increasing level of exposure. It must be noted that evidence of lack of carcinogenicity obtained from several epidemiological studies can apply only to the type(s) of cancer studied, to the exposure levels reported and the timing and route of exposure studied, to the intervals between first exposure and disease onset observed in these studies, and to the general population(s) studied (i.e. there may be susceptible subpopulations or life stages). Experience from studies of cancer in humans indicates that the period from first exposure to the development of clinical cancer is sometimes longer than 20 years; therefore, latency periods substantially shorter than about 30 years cannot provide evidence of lack of carcinogenicity. Furthermore, there may be critical windows of exposure, for example, as with diethylstilboestrol and clear cell adenocarcinoma of the cervix and vagina (IARC, 2012a).

3. Studies of cancer in experimental animals

Most human carcinogens that have been studied adequately for carcinogenicity in experimental animals have produced positive results in one or more animal species. For some agents, carcinogenicity in experimental animals was demonstrated before epidemiological studies identified their carcinogenicity in humans. Although this observation cannot establish that all agents that cause cancer in experimental animals also cause cancer in humans, it is biologically plausible that agents for which there is *sufficient evidence of carcinogenicity* in experimental animals (see Part B, Section 6b) present

a carcinogenic hazard to humans. Accordingly, in the absence of additional scientific information, such as strong evidence that a given agent causes cancer in experimental animals through a species-specific mechanism that does not operate in humans (see Part B, Sections 4 and 6; Capen et al., 1999; IARC, 2003), these agents are considered to pose a potential carcinogenic hazard to humans. The inference of potential carcinogenic hazard to humans does not imply tumour site concordance across species (Baan et al., 2019).

(a) Types of studies considered

Relevant studies of cancer in experimental animals are identified by using systematic review principles as described in Part A, further elaborated in the Instructions for Authors, and as detailed below. Consideration is given to all available long-term studies of cancer in experimental animals with the agent under review (or possibly metabolites or derivatives of the agent) (see Part A, Section 7) after a thorough evaluation of the study features (see Part B, Section 3b). Those studies that are judged to be irrelevant to the evaluation or judged to be inadequate (e.g. too short a duration, too few animals, poor survival; see below) may be omitted. Guidelines for conducting long-term carcinogenicity experiments have been published (e.g. OECD, 2018).

In addition to conventional long-term bioassays, alternative studies (e.g. in genetically engineered mouse models) may be considered in assessing carcinogenicity in experimental animals, also after a critical evaluation of the study features. For studies of certain exposures, such as viruses that typically only infect humans, use of such specialized experimental animal models may be particularly important; models include genetically engineered mice with targeted expression of viral genes to tissues from which human cancers arise, as well as humanized mice implanted with the human cells usually infected by the virus.

Other types of studies can provide supportive evidence. These include: experiments in which the agent was administered in the presence of factors that modify carcinogenic effects (e.g. initiation–promotion studies); studies in which the end-point was not cancer but a defined precancerous lesion; and studies of cancer in non-laboratory animals (e.g. companion animals) exposed to the agent.

(b) Study evaluation

Considerations of importance in the interpretation and evaluation of a particular study include: (i) whether the agent was clearly characterized, including the nature and extent of impurities and contaminants and the stability of the agent, and, in the case of mixtures, whether the sample characterization was adequately reported; (ii) whether the dose was monitored adequately, particularly in inhalation experiments; (iii) whether the doses, duration and frequency of treatment, duration of observation, and route of exposure were appropriate; (iv) whether appropriate experimental animal species and strains were evaluated; (v) whether there were adequate numbers of animals per group; (vi) whether animals were allocated randomly to groups; (vii) whether the body weight, food and water consumption, and survival of treated animals were affected by any factors other than the test agent; (viii) whether the histopathology review was adequate; and (ix) whether the data were reported and analysed adequately.

(c) Outcomes and statistical analyses

An assessment of findings of carcinogenicity in experimental animals involves consideration of (i) study features such as route, doses, schedule and duration of exposure, species, strain (including genetic background where applicable), sex, age, and duration of follow-up; (ii) the

spectrum of neoplastic response, from pre-neoplastic lesions and benign tumours to malignant neoplasms; (iii) the incidence, latency, severity, and multiplicity of neoplasms and pre-neoplastic lesions; (iv) the consistency of the results for a specific target organ or organs across studies of similar design; and (v) the possible role of modifying factors (e.g. diet, infection, stress).

Key factors for statistical analysis include: (i) number of animals studied and number examined histologically, (ii) number of animals with a given tumour type or lesion, and (iii) duration of survival.

Benign tumours may be combined with malignant tumours in the assessment of tumour incidence when (a) they occur together with and originate from the same cell type as malignant tumours in an organ or tissue in a particular study and (b) they appear to represent a stage in the progression to malignancy (Huff et al., 1989). The occurrence of lesions presumed to be pre-neoplastic may in certain instances aid in assessing the biological plausibility of any neoplastic response observed.

Evidence of an increased incidence of neoplasms with increasing level of exposure strengthens the inference of a causal association between the exposure and the development of neoplasms. The form of the dose–response relationship can vary widely, including non-linearity, depending on the particular agent under study and the target organ. The dose–response relationship can also be affected by differences in survival among the treatment groups.

The statistical methods used should be clearly stated and should be the generally accepted techniques refined for this purpose (Peto et al., 1980; Gart et al., 1986; Portier & Bailer, 1989; Bieler & Williams, 1993). The choice of the most appropriate statistical method requires consideration of whether there are differences in survival among the treatment groups; for example, reduced survival because of non-tumour-related mortality can preclude the occurrence of tumours later in life and a survival-adjusted analysis would be warranted. When detailed information on survival is not available, comparisons of the proportions of tumour-bearing animals among the effective number of animals (alive at the time that the first tumour was discovered) can be useful when significant differences in survival occur before tumours appear. The lethality of the tumour also requires consideration: for rapidly fatal tumours, the time of death provides an indication of the time of tumour onset and can be assessed using life-table methods; non-fatal or incidental tumours that do not affect survival can be assessed using methods such as the Mantel–Haenszel test for changes in tumour prevalence. Because tumour lethality is often difficult to determine, methods such as the poly-*k* test that do not require such information can also be used. When results are available on the number and size of tumours seen in experimental animals (e.g. papillomas on mouse skin, liver tumours observed through nuclear magnetic resonance tomography), other, more complicated statistical procedures may be needed (Sherman et al., 1994; Dunson et al., 2003).

The concurrent control group is generally the most appropriate comparison group for statistical analysis; however, for uncommon tumours, the analysis may be improved by considering historical control data, particularly when between-study variability is low. Historical controls should be selected to resemble the concurrent controls as closely as possible with respect to species, sex, and strain, as well as other factors, such as basal diet and general laboratory environment, which may affect tumour response rates in control animals (Haseman et al., 1984; Fung et al., 1996; Greim et al., 2003). It is generally not appropriate to discount a tumour response that is significantly increased compared with concurrent controls by arguing that it falls within the range of historical controls.

Meta-analyses and pooled analyses may be appropriate when the experimental protocols are sufficiently similar.

4. Mechanistic evidence

Mechanistic data may provide evidence of carcinogenicity and may also help in assessing the relevance and importance of findings of cancer in experimental animals and in humans (Guyton et al., 2009; Parkkinen et al., 2018) (see Part B, Section 6). Mechanistic studies have gained in prominence, increasing in their volume, diversity, and relevance to cancer hazard evaluation, whereas studies pertinent to other streams of evidence evaluated in the *Monographs* (i.e. studies of cancer in humans and lifetime cancer bioassays in rodents) may only be available for a fraction of agents to which humans are currently exposed (Guyton et al., 2009, 2018). Mechanistic studies and data are identified, screened, and evaluated for quality and importance to the evaluation by using systematic review principles as described in Part A, further elaborated in the Instructions for Authors, and as detailed below.

The Working Group's synthesis reflects the extent of available evidence, summarizing groups of included studies with an emphasis on characterizing consistencies or differences in results within and across experimental designs. Greater emphasis is given to informative mechanistic evidence from human-related studies than to that from other experimental test systems, and gaps are identified. Tabulation of data may facilitate this review. The specific topics addressed in the evidence synthesis are described below.

(a) Absorption, distribution, metabolism, and excretion

Studies of absorption, distribution, metabolism, and excretion in mammalian species are addressed in a summary fashion; exposure characterization is addressed in Part B, Section 1. The Working Group describes the metabolic fate of the agent in mammalian species, noting the metabolites that have been identified and their chemical reactivity. A metabolic schema may indicate the relevant metabolic pathways and products and whether supporting evidence is from studies in humans and/or studies in experimental animals. Evidence on other adverse effects that indirectly confirm absorption, distribution, and/or metabolism at tumour sites is briefly summarized when direct evidence is sparse.

(b) Evidence relevant to key characteristics of carcinogens

A review of Group 1 human carcinogens classified up to and including *IARC Monographs* Volume 100 revealed several issues relevant to improving the evaluation of mechanistic evidence for cancer hazard identification (Smith et al., 2016). First, it was noted that human carcinogens often share one or more characteristics that are related to the multiple mechanisms by which agents cause cancer. Second, different human carcinogens may exhibit a different spectrum of these key characteristics and operate through distinct mechanisms. Third, for many carcinogens evaluated before Volume 100, few data were available on some mechanisms of recognized importance in carcinogenesis, such as epigenetic alterations (Herceg et al., 2013). Fourth, there was no widely accepted method to search systematically for relevant mechanistic evidence, resulting in a lack of uniformity in the scope of mechanistic topics addressed across *IARC Monographs* evaluations.

To address these challenges, the key characteristics of human carcinogens were introduced to facilitate systematic consideration of mechanistic evidence in *IARC Monographs* evaluations (Smith et al., 2016; Guyton et al., 2018). The key characteristics described by Smith et al. (2016) (see Table 3), such as "is genotoxic", "is

Table 3 The key characteristics of carcinogens

	Ten key characteristics of carcinogens
1.	Is electrophilic or can be metabolically activated to an electrophile
2.	Is genotoxic
3.	Alters DNA repair or causes genomic instability
4.	Induces epigenetic alterations
5.	Induces oxidative stress
6.	Induces chronic inflammation
7.	Is immunosuppressive
8.	Modulates receptor-mediated effects
9.	Causes immortalization
10.	Alters cell proliferation, cell death, or nutrient supply

From Smith et al. (2016).

immunosuppressive", or "modulates receptor-mediated effects", are based on empirical observations of the chemical and biological properties associated with the human carcinogens identified by the *IARC Monographs* programme up to and including Volume 100. The list of key characteristics and associated end-points may evolve, based on the experience of their application and as new human carcinogens are identified. Key characteristics are distinct from the "hallmarks of cancer", which relate to the properties of cancer cells (Hanahan & Weinberg, 2000, 2011). Key characteristics are also distinct from hypothesized mechanistic pathways, which describe a sequence of biological events postulated to occur during carcinogenesis. As such, the evaluation approach based on key characteristics, outlined below, "avoids a narrow focus on specific pathways and hypotheses and provides for a broad, holistic consideration of the mechanistic evidence" (National Academies of Sciences, Engineering, and Medicine, 2017).

Studies in exposed humans and in human primary cells or tissues that incorporate end-points relevant to key characteristics of carcinogens are emphasized when available. For each key characteristic with adequate evidence for evaluation, studies are grouped according to whether they involve (a) humans or human primary cells or tissues or (b) experimental systems; further organization (as appropriate) is by end-point (e.g. DNA damage), duration, species, sex, strain, and target organ as well as strength of study design. Studies investigating susceptibility related to key characteristics of carcinogens (e.g. of genetic polymorphisms, or in genetically engineered animals) can be highlighted and may provide additional support for conclusions on the strength of evidence. Findings relevant to a specific tumour type may be noted.

(c) Other relevant evidence

Other informative evidence may be described when it is judged by the Working Group to be relevant to an evaluation of carcinogenicity and to be of sufficient importance to affect the overall evaluation. Quantitative structure–activity information, such as on specific chemical and/or biological features or activities (e.g. electrophilicity, molecular docking with receptors), may be informative. In addition, evidence that falls outside of the recognized key characteristics of carcinogens, reflecting emerging knowledge or important novel scientific developments on carcinogen mechanisms, may also be included. Available evidence relevant to criteria provided in authoritative publications (e.g. Capen et al.,

1999; IARC, 2003) on thyroid, kidney, urinary bladder, or other tumours in experimental animals induced by mechanisms that do not operate in humans is also described.

(d) Study quality and importance to the evaluation

Based on formal considerations of the quality of the studies (e.g. design, methodology, and reporting of results), the Working Group may give greater weight to some included studies.

For observational and other studies in humans, the quality of study design, exposure assessment, and assay accuracy and precision are considered, in collaboration with the Working Group members reviewing exposure characterization and studies of cancer in humans, as are other important factors, including those described above for evaluation of epidemiological evidence (García-Closas et al., 2006, 2011; Vermeulen et al., 2018) (Part B, Sections 1 and 2).

In general, in experimental systems, studies of repeated doses and of chronic exposures are accorded greater importance than are studies of a single dose or time-point. Consideration is also given to factors such as the suitability of the dosing range, the extent of concurrent toxicity observed, and the completeness of reporting of the study (e.g. the source and purity of the agent, the analytical methods, and the results). Route of exposure is generally considered to be a less important factor in the evaluation of experimental studies, recognizing that the exposures and target tissues may vary across experimental models and in exposed human populations. Non-mammalian studies can be synthetically summarized when they are considered to be supportive of evidence in humans or higher organisms.

In vitro test systems can provide mechanistic insights, but important considerations include the limitations of the test system (e.g. in metabolic capabilities) as well as the suitability of a particular test article (i.e. because of physical and chemical characteristics) (Hopkins et al., 2004). For studies on some end-points, such as for traditional studies of mutations in bacteria and in mammalian cells, formal guidelines, including those from the Organisation for Economic Co-operation and Development, may be informative in conducting the quality review (OECD, 1997, 2016a, b). However, existing guidelines will not generally cover all relevant assays, even for genotoxicity. Possible considerations when evaluating the quality of in vitro studies encompass the methodology and design (e.g. the end-point and test method, the number of replicate samples, the suitability of the concentration range, the inclusion of positive and negative controls, and the assessment of cytotoxicity) as well as reporting (e.g. of the source and purity of the agent, and of the analytical methods and results). High-content and high-throughput in vitro data can serve as an additional or supportive source of mechanistic evidence (Chiu et al., 2018; Guyton et al., 2018), although large-scale screening programmes measuring a variety of end-points were designed to evaluate large chemical libraries in order to prioritize chemicals for additional toxicity testing rather than to identify the hazard of a specific chemical or chemical group.

The synthesis is focused on the evidence that is most informative for the overall evaluation. In this regard, it is of note that some human carcinogens exhibit a single or primary key characteristic, evidence of which has been influential in their cancer hazard classifications. For instance, ethylene oxide is genotoxic (IARC, 1994), 2,3,7,8-tetrachlorodibenzo-*para*-dioxin modulates receptor-mediated effects (IARC, 1997), and etoposide alters DNA repair (IARC, 2012a). Similarly, oncogenic viruses cause immortalization, and certain drugs are, by design, immunosuppressive (IARC, 2012a, b). Because non-carcinogens can also induce oxidative stress, this key characteristic should be interpreted

with caution unless it is found in combination with other key characteristics (Guyton et al., 2018). Evidence for a group of key characteristics can strengthen mechanistic conclusions (e.g. "induces oxidative stress" together with "is electrophilic or can be metabolically activated to an electrophile", "induces chronic inflammation", and "is immunosuppressive"); see, for example, 1-bromopropane (IARC, 2018).

5. Summary of data reported

(a) Exposure characterization

Exposure data are summarized to identify the agent and describe its production, use, and occurrence. Information on exposure prevalence and intensity in different settings, including geographical patterns and time trends, may be included. Exposure assessment methods used in key epidemiological studies reviewed by the Working Group are described and evaluated.

(b) Cancer in humans

Results of epidemiological studies pertinent to an evaluation of carcinogenicity in humans are summarized. The overall strengths and limitations of the epidemiological evidence base are highlighted to indicate how the evaluation was reached. The target organ(s) or tissue(s) in which a positive association between the agent and cancer was observed are identified. Exposure–response and other quantitative data may be summarized when available. When the available epidemiological studies pertain to a mixed exposure, process, occupation, or industry, the Working Group seeks to identify the specific agent considered to be most likely to be responsible for any excess risk. The evaluation is focused as narrowly as the available data permit.

(c) Cancer in experimental animals

Results pertinent to an evaluation of carcinogenicity in experimental animals are summarized to indicate how the evaluation was reached. For each animal species, study design, and route of administration, there is a statement about whether an increased incidence, reduced latency, or increased severity or multiplicity of neoplasms or pre-neoplastic lesions was observed, and the tumour sites are indicated. Special conditions resulting in tumours, such as prenatal exposure or single-dose experiments, are mentioned. Negative findings, inverse relationships, dose–response patterns, and other quantitative data are also summarized.

(d) Mechanistic evidence

Results pertinent to an evaluation of the mechanistic evidence on carcinogenicity are summarized to indicate how the evaluation was reached. The summary encompasses the informative studies on absorption, distribution, metabolism, and excretion; on the key characteristics with adequate evidence for evaluation; and on any other aspects of sufficient importance to affect the overall evaluation, including on whether the agent belongs to a class of agents for which one or more members have been classified as carcinogenic or probably carcinogenic to humans, and on criteria with respect to tumours in experimental animals induced by mechanisms that do not operate in humans. For each topic addressed, the main supporting findings are highlighted from exposed humans, human cells or tissues, experimental animals, or in vitro systems. When mechanistic studies are available in exposed humans, the tumour type or target tissue studied may be specified. Gaps in the evidence are indicated (i.e. if no studies were available in exposed humans, in in vivo systems, etc.). Consistency or differences of effects across different experimental systems are emphasized.

6. Evaluation and rationale

Consensus evaluations of the strength of the evidence of cancer in humans, the evidence of cancer in experimental animals, and the mechanistic evidence are made using transparent criteria and defined descriptive terms. The Working Group then develops a consensus overall evaluation of the strength of the evidence of carcinogenicity for each agent under review.

An evaluation of the strength of the evidence is limited to the agents under review. When multiple agents being evaluated are considered by the Working Group to be sufficiently closely related, they may be grouped together for the purpose of a single and unified evaluation of the strength of the evidence.

The framework for these evaluations, described below, may not encompass all factors relevant to a particular evaluation of carcinogenicity. After considering all relevant scientific findings, the Working Group may exceptionally assign the agent to a different category than a strict application of the framework would indicate, while providing a clear rationale for the overall evaluation.

When there are substantial differences of scientific interpretation among the Working Group members, the overall evaluation will be based on the consensus of the Working Group. A summary of the alternative interpretations may be provided, together with their scientific rationale and an indication of the relative degree of support for each alternative.

The categories of the classification refer to the strength of the evidence that an exposure is carcinogenic and not to the risk of cancer from particular exposures. The terms *probably carcinogenic* and *possibly carcinogenic* have no quantitative significance and are used as descriptors of different strengths of evidence of carcinogenicity in humans; *probably carcinogenic* signifies a greater strength of evidence than *possibly carcinogenic*.

(a) Carcinogenicity in humans

Based on the principles outlined in Part B, Section 2, the evidence relevant to carcinogenicity from studies in humans is classified into one of the following categories:

***Sufficient evidence of carcinogenicity*:** A causal association between exposure to the agent and human cancer has been established. That is, a positive association has been observed in the body of evidence on exposure to the agent and cancer in studies in which chance, bias, and confounding were ruled out with reasonable confidence.

***Limited evidence of carcinogenicity*:** A causal interpretation of the positive association observed in the body of evidence on exposure to the agent and cancer is credible, but chance, bias, or confounding could not be ruled out with reasonable confidence.

***Inadequate evidence regarding carcinogenicity*:** The available studies are of insufficient quality, consistency, or statistical precision to permit a conclusion to be drawn about the presence or the absence of a causal association between exposure and cancer, or no data on cancer in humans are available. Common findings that lead to a determination of inadequate evidence of carcinogenicity include: (a) there are no data available in humans; (b) there are data available in humans, but they are of poor quality or informativeness; and (c) there are studies of sufficient quality available in humans, but their results are inconsistent or otherwise inconclusive.

***Evidence suggesting lack of carcinogenicity*:** There are several high-quality studies covering the full range of levels of exposure that humans are known to encounter, which are mutually consistent in not showing a positive association between exposure to the agent and the studied cancers at any

observed level of exposure. The results from these studies alone or combined should have narrow confidence intervals with an upper limit below or close to the null value (e.g. a relative risk of unity). Bias and confounding were ruled out with reasonable confidence, and the studies were considered informative. A conclusion of *evidence suggesting lack of carcinogenicity* is limited to the cancer sites, populations and life stages, conditions and levels of exposure, and length of observation covered by the available studies. In addition, the possibility of a very small risk at the levels of exposure studied can never be excluded.

When there is *sufficient evidence*, a separate sentence identifies the target organ(s) or tissue(s) for which a causal interpretation has been established. When there is *limited evidence*, a separate sentence identifies the target organ(s) or tissue(s) for which a positive association between exposure to the agent and the cancer(s) was observed in humans. When there is *evidence suggesting lack of carcinogenicity*, a separate sentence identifies the target organ(s) or tissue(s) where evidence of lack of carcinogenicity was observed in humans. Identification of a specific target organ or tissue as having *sufficient evidence* or *limited evidence* or *evidence suggesting lack of carcinogenicity* does not preclude the possibility that the agent may cause cancer at other sites.

(b) Carcinogenicity in experimental animals

The evidence relevant to carcinogenicity from studies in experimental animals is classified into one of the following categories:

***Sufficient evidence of carcinogenicity*:** A causal relationship has been established between exposure to the agent and cancer in experimental animals based on an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories and/or under different protocols. An increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices (GLP), can also provide *sufficient evidence*.

Exceptionally, a single study in one species and sex may be considered to provide *sufficient evidence of carcinogenicity* when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour, or age at onset, or when there are marked findings of tumours at multiple sites.

***Limited evidence of carcinogenicity*:** The data suggest a carcinogenic effect but are limited for making a definitive evaluation because, for example, (a) the evidence of carcinogenicity is restricted to a single experiment and does not meet the criteria for *sufficient evidence*; (b) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; (c) the agent increases tumour multiplicity or decreases tumour latency but does not increase tumour incidence; (d) the evidence of carcinogenicity is restricted to initiation–promotion studies; (e) the evidence of carcinogenicity is restricted to observational studies in non-laboratory animals (e.g. companion animals); or (f) there are unresolved questions about the adequacy of the design, conduct, or interpretation of the available studies.

***Inadequate evidence regarding carcinogenicity*:** The studies cannot be interpreted as showing either the presence or the absence

of a carcinogenic effect because of major qualitative or quantitative limitations, or no data are available on cancer in experimental animals.

***Evidence suggesting lack of carcinogenicity*:** Well-conducted studies (e.g. conducted under GLP) involving both sexes of at least two species are available showing that, within the limits of the tests used, the agent was not carcinogenic. The conclusion of *evidence suggesting lack of carcinogenicity* is limited to the species, tumour sites, age at exposure, and conditions and levels of exposure covered by the available studies.

(c) Mechanistic evidence

Based on the principles outlined in Part B, Section 4, the mechanistic evidence is classified into one of the following categories:

***Strong mechanistic evidence*:** Results in several different experimental systems are consistent, and the overall mechanistic database is coherent. Further support can be provided by studies that demonstrate experimentally that the suppression of key mechanistic processes leads to the suppression of tumour development. Typically, a substantial number of studies on a range of relevant end-points are available in one or more mammalian species. Quantitative structure–activity considerations, in vitro tests in non-human mammalian cells, and experiments in non-mammalian species may provide corroborating evidence but typically do not in themselves provide strong evidence. However, consistent findings across a number of different test systems in different species may provide strong evidence.

Of note, "strong" relates not to potency but to strength of evidence. The classification applies to three distinct topics:

(a) Strong evidence that the agent belongs, based on mechanistic considerations, to a class of agents for which one or more members have been classified as carcinogenic or probably carcinogenic to humans. The considerations can go beyond quantitative structure–activity relationships to incorporate similarities in biological activity relevant to common key characteristics across dissimilar chemicals (e.g. based on molecular docking, –omics data).

(b) Strong evidence that the agent exhibits key characteristics of carcinogens. In this case, three descriptors are possible:

1. The strong evidence is in exposed humans. Findings relevant to a specific tumour type may be informative in this determination.
2. The strong evidence is in human primary cells or tissues. Specifically, the strong findings are from biological specimens obtained from humans (e.g. ex vivo exposure), from human primary cells, and/or, in some cases, from other humanized systems (e.g. a human receptor or enzyme).
3. The strong evidence is in experimental systems. This may include one or a few studies in human primary cells and tissues.

(c) Strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans. Certain results in experimental animals (see Part B, Section 6b) would be discounted, according to relevant criteria and considerations in authoritative publications (e.g. Capen et al., 1999; IARC, 2003). Typically, this classification would not apply when there is strong mechanistic evidence that the agent exhibits key characteristics of carcinogens.

***Limited mechanistic evidence*:** The evidence is suggestive, but, for example, (a) the studies cover a narrow range of experiments, relevant end-points, and/or species; (b) there are unexplained inconsistencies in the studies of similar design; and/or (c) there is unexplained incoherence across studies of different end-points or in different experimental systems.

***Inadequate mechanistic evidence*:** Common findings that lead to a determination of inadequate mechanistic evidence include: (a) few or no data are available; (b) there are unresolved questions about the adequacy of the design, conduct, or interpretation of the studies; (c) the available results are negative.

(d) Overall evaluation

Finally, the bodies of evidence included within each stream of evidence are considered as a whole, in order to reach an overall evaluation of the carcinogenicity of the agent to humans. The three streams of evidence are integrated and the agent is classified into one of the following categories (see Table 4), indicating that the Working Group has established that:

The agent is carcinogenic to humans (Group 1)

This category applies whenever there is *sufficient evidence of carcinogenicity* in humans.

In addition, this category may apply when there is both *strong evidence in exposed humans that the agent exhibits key characteristics of carcinogens* and *sufficient evidence of carcinogenicity* in experimental animals.

The agent is probably carcinogenic to humans (Group 2A)

This category generally applies when the Working Group has made at least *two of the following* evaluations, *including at least one* that involves either exposed humans or human cells or tissues:

- *Limited evidence of carcinogenicity* in humans,
- *Sufficient evidence of carcinogenicity* in experimental animals,
- *Strong evidence that the agent exhibits key characteristics of carcinogens.*

If there is *inadequate evidence regarding carcinogenicity* in humans, there should be *strong evidence in human cells or tissues that the agent exhibits key characteristics of carcinogens*. If there is *limited evidence of carcinogenicity in humans*, then the second individual evaluation may be from experimental systems (i.e. s*ufficient evidence of carcinogenicity* in experimental animals or *strong evidence in experimental systems that the agent exhibits key characteristics of carcinogens).*

Additional considerations apply when there is *strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans* for one or more tumour sites. Specifically, the remaining tumour sites should still support an evaluation of *sufficient evidence in experimental animals* in order for this evaluation to be used to support an overall classification in Group 2A.

Separately, this category generally applies if there is *strong evidence that the agent belongs, based on mechanistic considerations, to a class of agents for which one or more members have been classified in Group 1 or Group 2A*.

The agent is possibly carcinogenic to humans (Group 2B)

This category generally applies when only one of the following evaluations has been made by the Working Group:

- *Limited evidence of carcinogenicity* in humans,
- *Sufficient evidence of carcinogenicity* in experimental animals,

Table 4 Integration of streams of evidence in reaching overall classifications (the evidence in *bold italic* represents the basis of the overall evaluation)

Stream of evidence			Classification based on strength of evidence
Evidence of cancer in humans[a]	**Evidence of cancer in experimental animals**	**Mechanistic evidence**	
Sufficient	Not necessary	Not necessary	**Carcinogenic to humans (Group 1)**
Limited or Inadequate	***Sufficient***	***Strong (b)(1) (exposed humans)***	
Limited	***Sufficient***	Strong (b)(2–3), Limited, or Inadequate	**Probably carcinogenic to humans (Group 2A)**
Inadequate	***Sufficient***	***Strong (b)(2) (human cells or tissues)***	
Limited	Less than Sufficient	***Strong (b)(1–3)***	
Limited or Inadequate	Not necessary	***Strong (a) (mechanistic class)***	
Limited	Less than Sufficient	Limited or Inadequate	**Possibly carcinogenic to humans (Group 2B)**
Inadequate	***Sufficient***	Strong (b)(3), Limited, or Inadequate	
Inadequate	Less than Sufficient	***Strong b(1–3)***	
Limited	***Sufficient***	***Strong (c) (does not operate in humans)***[b]	
Inadequate	***Sufficient***	***Strong (c) (does not operate in humans)***[b]	**Not classifiable as to its carcinogenicity to humans (Group 3)**
All other situations not listed above			

[a] Human cancer(s) with highest evaluation

[b] The *strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans* must specifically be for the tumour sites supporting the classification of *sufficient evidence in experimental animals*.

• *Strong evidence that the agent exhibits key characteristics of carcinogens.*

Because this category can be based on evidence from studies in experimental animals alone, there is **no** requirement that the strong mechanistic evidence be in exposed humans or in human cells or tissues. This category may be based on *strong evidence in experimental systems that the agent exhibits key characteristics of carcinogens.*

As with Group 2A, additional considerations apply when there is *strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans* for one or more tumour sites. Specifically, the remaining tumour sites should still support an evaluation of *sufficient evidence in experimental animals* in order for this evaluation to be used to support an overall classification in Group 2B.

The agent is not classifiable as to its carcinogenicity to humans (Group 3)

Agents that do not fall into any other group are generally placed in this category.

This includes the case when there is *strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans* for one or more tumour sites in experimental animals, the remaining tumour sites do not support an evaluation of *sufficient evidence in experimental animals*, and other categories are not supported by data from studies in humans and mechanistic studies.

An evaluation in Group 3 is not a determination of non-carcinogenicity or overall safety. It often means that the agent is of unknown carcinogenic potential and that there are significant gaps in research.

If the evidence suggests that the agent exhibits no carcinogenic activity, either through *evidence suggesting lack of carcinogenicity* in both humans and experimental animals, or through

evidence suggesting lack of carcinogenicity in experimental animals complemented by strong negative mechanistic evidence in assays relevant to human cancer, then the Working Group may add a sentence to the evaluation to characterize the agent as well-studied and without evidence of carcinogenic activity.

(e) Rationale

The reasoning that the Working Group used to reach its evaluation is summarized so that the basis for the evaluation offered is transparent. This section integrates the major findings from studies of cancer in humans, cancer in experimental animals, and mechanistic evidence. It includes concise statements of the principal line(s) of argument that emerged in the deliberations of the Working Group, the conclusions of the Working Group on the strength of the evidence for each stream of evidence, an indication of the body of evidence that was pivotal to these conclusions, and an explanation of the reasoning of the Working Group in making its evaluation.

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GENERAL REMARKS

This one-hundred-and-twenty-ninth volume of the *IARC Monographs* contains evaluations of the carcinogenic hazard to humans of gentian violet, leucogentian violet, malachite green, leucomalachite green, and CI Direct Blue 218. Due to the coronavirus disease (COVID-19) pandemic, this meeting, which was scheduled to be held in Lyon, France, was held remotely.

None of these agents have been evaluated previously by the *IARC Monographs* programme.

The Advisory Group to Recommend Priorities for the *IARC Monographs* that met in 2019 recommended that gentian violet, malachite green, and leucomalachite green be evaluated with high priority, and CI Direct Blue 218 with medium priority (Marques et al., 2019). A summary of the findings of this volume appears in *The Lancet Oncology* (LeCurieux et al., 2021).

Paucity of exposure data

For all the five dyes in this volume, the Working Group observed substantial data gaps regarding production and use, as well as environmental and occupational exposure levels. These data gaps are particularly notable in low- and middle-income countries but also exist in high-income countries. The Working Group has noted in the monograph on CI Direct Blue 218 that these data gaps were especially surprising given that CI Direct Blue 218 is listed by the Organisation for Economic Co-operation and Development (for the year 2007) as a High Production Volume chemical (OECD, 2009), and has widespread potential for occupational exposure during the manufacturing process (synthesis, processing, packaging, transportation, or maintenance and clean-up), during the application of the dye on products, and also during any additional processing of dyed products that results in particle formation (NIOSH, 1983).

Dye purity

The Working Group noted that the poor purity of all the dyes considered in the present volume, but especially CI Direct Blue 218, has been shown to be an important drawback to interpretation of the results of the available studies. If more experiments were performed in the future, dyes of a high purity (> 95%, if possible) should be tested to ensure that any effect observed can be attributed to the dye itself and not to other compounds (i.e. impurities) present in the sample.

Metabolism and mutagenicity of CI Direct Blue 218

Among the evidence gaps identified in this volume was whether CI Direct Blue 218 is metabolized to benzidine (classified as *carcinogenic to humans*, Group 1), or the benzidine congeners 3,3′-dihydroxybenzidine or 3,3′-dimethoxybenzidine. This gap was noted previously in *IARC Monographs* Volume 99, when CI Direct Blue 218 was not included in the classification of the agent "Dyes metabolized to benzidine" (as *carcinogenic to humans*, Group 1), in contrast to other dyes such as Direct Black 38, Direct Blue 6, and Direct Brown 95 (IARC, 2010). The Working Group at that time suggested that future mechanistic studies should determine whether enzymatic reduction of CI Direct Blue 218 would generate the benzidine congener 3,3′-dimethoxybenzidine. Another evidence gap was the lack of informative studies elucidating the mutagenicity of CI Direct Blue 218. The Working Group considered that mechanistic studies are also warranted to test CI Direct Blue 218 in assays for gene mutation in the presence of endogenous metabolic activation with *Salmonella typhimurium* strains YG1041 or YG1024 that are particularly sensitive to aromatic amines.

Distinguishing between various salts of malachite green in exposure characterization data

The dye malachite green occurs as a chloride but is also available as an oxalate and as other salts, which are each used in various amounts for different and common applications. While some information was available to the Working Group regarding specific applications for the different chemical forms of malachite green, all are often referred to interchangeably by the general term "malachite green". This is particularly true in the literature on exposure characterization (e.g. reports of concentrations of malachite green residue measured in various matrices), in which the distinction between the different chemical forms of malachite green is often not made.

Environmental transformation of gentian violet and malachite green to and from their leucometabolites

In the environment, malachite green is transformed via a reversible reaction (reduction↔oxidation) under anaerobic conditions into leucomalachite green. In the atmosphere and in water, malachite green and gentian violet may undergo photodegradation into leucomalachite green and leucogentian violet, respectively. In vivo, enzymatic transformation of malachite green and gentian violet into their corresponding leucometabolites is well documented, although data on humans are scarce. Consequently, co-occurrence of each dye with its leucometabolite is likely.

Data from high-throughput screening assays

The analysis of the in vitro bioactivity of gentian violet, malachite green chloride, malachite green oxalate, and leucomalachite green was informed by data from high-throughput screening assays generated by the Toxicity Testing in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) research programmes of the government of the USA (Thomas et al., 2018). All compounds were considered active in a variety of the assay end-points mapped to the following key characteristics of carcinogens: induces oxidative stress, modulates receptor-mediated effects, and

alters cell proliferation, cell death, or nutrient supply. Specifically, gentian violet and malachite green oxalate were considered active in most of the "is genotoxic" assay end-points, and malachite green chloride was considered active in all the "induces epigenetic alterations" assay end-points. The mapping of assay end-points to each key characteristic follows that described in *IARC Monographs* Volume 123 (IARC, 2019). All ToxCast/Tox21 data were downloaded from the United States Environmental Protection Agency CompTox Chemicals Dashboard 10th Release (US EPA, 2021) on 2–19 October 2020 or on 24 February 2021 (malachite green oxalate). These programmes are constantly being improved and new assays are added over time. However, at present, the general lack of metabolic activation and the small number of genotoxicity assays in these high-throughput screening programmes restrict their value in determining whether a chemical is genotoxic as part of an assessment of carcinogenicity.

Scope of the systematic review

Standardized searches of the PubMed database (NCBI, 2021) were conducted for the agent and for each outcome (cancer in humans, cancer in experimental animals, and mechanistic evidence, including the key characteristics of carcinogens). The literature trees for the agent, including the full set of search terms for the agent name and each outcome type, are available online.[1]

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[1] The literature trees for the present volume are available at: https://hawcproject.iarc.who.int/assessment/626/ (gentian violet), https://hawcproject.iarc.who.int/assessment/655/ (leucogentian violet), https://hawcproject.iarc.who.int/assessment/648/ (malachite green), https://hawcproject.iarc.who.int/assessment/649/(leucomalachite green), and https://hawcproject.iarc.who.int/assessment/652/ (CI Direct Blue 218).

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GENTIAN VIOLET AND LEUCOGENTIAN VIOLET

1. Exposure Characterization

1.1 Identification of the agent

Gentian violet is a cationic triphenylmethane dye. Leucogentian violet, the leuco base or reduced form of gentian violet, is formed by the chemical or enzymatic reduction of gentian violet. Gentian violet and its leuco base are susceptible to oxidation–reduction and demethylation reactions.

1.1.1 Gentian violet

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 548-62-9

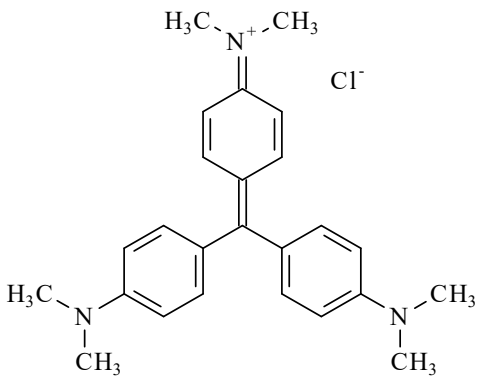
Chem. Abstr. Serv. name: *N*-[4-[bis[4-(dimethylamino)phenyl]methylene]-2,5-cyclohexadien-1-ylidene]-*N*-methylmethanaminium chloride (1 : 1)

EC No.: 208-953-6

IUPAC systematic name: [4-[bis[4-(dimethylamino)phenyl]methylidene]cyclo-hexa-2,5-dien-1-ylidene]-dimethylazanium chloride; (4-[4,4-bis(dimethylamino)benzhydrylidene]cyclohexa-2,5-dien-1-ylidene)dimethylammonium chloride; tris(4-(dimethylamino)phenyl)methylium chloride

Synonyms: CI Basic Violet 3, CI 42555, basic violet, crystal violet, hexamethyl-*para*-rosaniline chloride, methyl violet 10B, methylrosanilium chloride, aniline violet (ECHA, 2020a; NCBI, 2020).

(b) Structural and molecular formulae, and relative molecular mass



Molecular formula: $C_{25}H_{30}ClN_3$

Relative molecular mass: 407.98

(c) Chemical and physical properties of the pure substance

Description: green to very dark green powder; dark purple in solution

Boiling point: 631.92 °C (ECHA, 2020a)

Melting point: 205–215 °C (decomposes) (NCBI, 2013); 198 °C (ECHA, 2020a)

Density: 1.19 g/cm^3 at 20 °C (OEHHA, 2019)

Solubility: 4000 mg/L at 25 °C, and 10–50 g/L at 27 °C and pH 3.07, in water (ECHA, 2020a); soluble in ethanol and chloroform (NCBI, 2013)

Vapour pressure: 1.02 × 10^{-13} mm Hg [1.36 × 10^{-14} kPa] at 25 °C (estimated) (NCBI, 2013); 0 Pa at 25 °C (ECHA, 2020a)

Auto-ignition temperature: > 190 °C (United States Pharmacopeia, 2014)

Stability and reactivity: stable under normal conditions; light-sensitive; incompatible with strong oxidizing agents, reducing agents, and strong acids (United States Pharmacopeia, 2014; Mani & Bharagava, 2016)

Octanol/water partition coefficient (P): log K_{ow} = 0.51 (NCBI, 2013)

Henry's law constant: 3.06 × 10^{-16} atm m^3 mol^{-1} [3.10 × 10^{-10} Pa m^3 mol^{-1}] (estimated) at 25 °C (NLM, 2020)

Ultraviolet maximum: 590 nm (water) (NCBI, 2013).

(d) Impurities

Gentian violet is composed primarily of hexamethyl-*para*-rosaniline (crystal violet) with impurities of pentamethyl-*para*-rosaniline and tetramethyl-*para*-rosaniline (Cooksey, 2017). The purity of gentian violet may range from > 76% to < 90% (w/w) (ECHA, 2012). The composition of commercial gentian violet is typically > 96% hexamethyl-*para*-rosaniline, < 4% pentamethyl-*para*-rosaniline, < 4% tetramethyl-*para*-rosaniline, and a trace amount of trimethyl-*para*-rosaniline (OEHHA, 2019). Unreacted reagents such as Michler's ketone or Michler's base may also be present (Cooksey, 2017).

1.1.2 Leucogentian violet

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 603-48-5

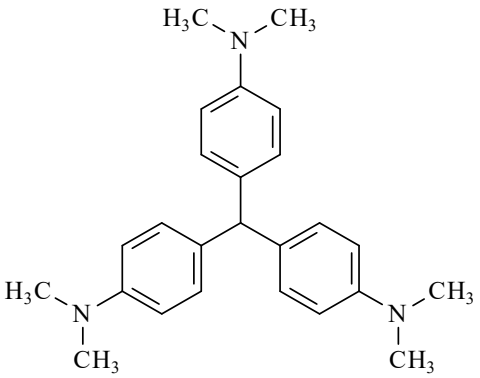
Chem. Abstr. Serv. name: leucocrystal violet

EC No.: 210-043-9

IUPAC systematic name: 4-[bis[4-(dimethylamino)phenyl]methyl]-*N*,*N*-dimethylaniline

Synonyms: leucocrystal violet, leuco Basic Violet 3, crystal violet leucobase, 4,4′,4″-tris(dimethylamino)triphenylmethane, 4,4′,4″-methylidynetris-*N*,*N*-dimethyl-benzenamine, 4,4′,4″-methylidynetris-*N*,*N*-dimethylaniline, tris[*para*-(dimethylamino)phenyl] methane, *N*,*N*,*N*′,*N*′,*N*″,*N*″-hexamethyl-4,4′,4″-methylidynetrianiline (NCBI, 2020).

(b) Structural and molecular formulae, and relative molecular mass



Molecular formula: $C_{25}H_{31}N_3$

Relative molecular mass: 373.53

(c) Chemical and physical properties of the pure substance

Description: white to very pale lavender powder

Boiling point: decomposition at 227.8 °C, before reaching the boiling point (ECHA, 2020b)

Melting point: 175–177 °C (NCBI, 2013); 176.8 °C (ECHA, 2020b)

Density: 1.141 g/cm^3 at 19.6 °C (ECHA, 2020b)

Solubility: 1.3 mg/L at 20 °C and pH 7.4–8.7 in water (ECHA, 2020b); 0.6 mg/mL in ethanol (NCBI, 2013)

Vapour pressure: 1.95 × 10^{-5} Pa at 20 °C (ECHA, 2020b)

Stability and reactivity: stable under normal conditions; light- and air-sensitive; carbon and nitrogen oxides and hydrogen chloride may form from thermal decomposition (Chemical Book, 2017; ECHA, 2020b).

Octanol/water partition coefficient (P): log K_{ow} = 5.9 (ECHA, 2020b)

Ultraviolet maximum: 260 nm (Merck, 2021).

(d) Impurities

Leucogentian violet is available with a purity ranging from 98% to > 99%.

1.2 Production and use

1.2.1 Gentian violet

(a) Production process

Several methods are reported to produce gentian violet, each resulting in different compositions of the N-methylated *para*-rosaniline dye components (Gessner & Mayer, 2000; Cooksey, 2017). High-purity hexamethyl-*para*-rosaniline is produced from the condensation of *N*,*N*-dimethylaniline with Michler's ketone (4,4-bis(dimethylamino)benzophenone), which is an intermediate generated from the reaction of carbonyl dichloride (phosgene) with dimethylaniline (ECHA, 2012; Cooksey, 2017). Gentian violet can also be generated from the oxidation of leucogentian violet. In a "one-pot" reaction, leucogentian violet is produced from the condensation of *N*,*N*-dimethylaniline with formaldehyde, reaction with additional *N*,*N*-dimethylaniline, and oxidation in the presence of chloranil and a catalyst such as (dihydrodibenzotetraaza[14]annulene) iron, a vanadium or molybdenum compound, or a nitrous gas (Gessner & Mayer, 2000).

(b) Production volume

India and China are the largest producers of gentian violet (ECHA, 2012). [No information was found on production volumes in these countries.] In the USA, the production volumes of gentian violet were reported to be between > 500 000 and 1 million pounds [> 227–454 tonnes] per year in 1986 and 1990, and between 10 000 and 500 000 pounds [between 4.54 and 227 tonnes] per year in 1994, 1998, and 2002 (NCBI, 2013). Gentian violet is not produced in the European Union (EU), but the EU imports 210–230 tonnes of gentian violet per year (ECHA, 2012). In 2020, gentian violet was available from 36 suppliers in China, 15 suppliers in the USA, 9 suppliers in India, and 2 suppliers in Europe (Chemical Register, 2020a).

(c) Uses

Gentian violet has been in use for more than a century as a dye or pigment, biological stain, and topical antiseptic. It has numerous diverse applications because of its colouring and medicinal properties.

The deep blue-violet colour of gentian violet is used to dye numerous textiles including silk, cotton, wool, and nylon. Gentian violet is also used as a dye for paper and as a pigment for ballpoint pen and printer ink, paint, plastic, gasoline, varnish, oil, and wax (Gessner & Mayer, 2000; ECHA, 2012; Mani & Bharagava, 2016). Gentian violet can be used in food-packaging materials. Gentian violet is used to mark locations on the skin for body piercings (Skellie, 2020) and has also been used as a hair dye (Diamante et al.,

2009). [The Working Group noted that more than 100 posts and videos can be found online describing the use of gentian violet as a cheap source of home-made hair dye.]

Gentian violet is used in clinical and bacteriological laboratories as a stain for biological specimens, because it permits visualization of cellular and histological morphology, and to distinguish Gram-positive from Gram-negative bacteria; gentian violet is the primary purple stain used in the Gram staining method (Boyanova, 2018). It is used in surgery as a skin-marking dye (Granick et al., 1987) and in chromoendoscopy to stain the gastrointestinal tract to distinguish lesions from normal tissue (Singh et al., 2020). It is used to detect the presence of bacteria in countless biological assays and is also a pH indicator, with a colour change from yellow at pH 0.0 to blue-violet at pH 2.0 (Cooksey, 2017).

The antibacterial, antifungal, and anthelmintic properties of gentian violet have resulted in numerous applications in medicine (Maley & Arbiser, 2013). As a topical treatment, gentian violet is effective against Gram-positive bacteria including *Staphylococcus aureus* and *Streptococcus*, and has been used for the treatment of eczema, impetigo, and to prevent infection and promote the healing of wounds, burns, inflammation resulting from radiotherapy, and the umbilical stumps of infants. Importantly, gentian violet has been effectively used to treat methicillin-resistant *Staphylococcus aureus* infections of the dermis, middle ear, chest cavity, nostrils, and vascular grafts. For decades, washing affected areas with a dilute solution of gentian violet has been used to treat fungal infections; notably, oral, oesophageal, vulvovaginal (Watson & Calabretto, 2007), nipple, and catheter infections caused by *Candida*. Coating invasive medical devices (e.g. catheters) with gentian violet reduces the adherence of pathogenic organisms to biofilms, which may lead to infection. Finally, gentian violet has been used against protozoa (e.g. *Trypanosoma cruzi*, which cause blood transfusion-associated Chagas disease, and *Leishmania*), nematodes (pinworms), and some viral infections (oral hairy leukoplakia), and may contribute to the inhibition of angiogenesis and tumour growth (Maley & Arbiser, 2013). The antimicrobial properties of gentian violet also have applications in veterinary medicine. Gentian violet has been used in poultry feed to inhibit the growth of moulds and fungi, as a topical treatment for bacterial and fungal infections of the skin and eyes in livestock, and as an immersion-bath treatment for fungal and parasitic infections in fish, including *Ichthyophthirius multifiliis*, the protozoan that causes white spot disease (WHO, 2014a). Although gentian violet is restricted for use in aquaculture, it is a common treatment for diseases in aquarium fish. Gentian violet is also used in aerosol sprays, in combination with antibiotics or insecticides, for the treatment of skin and hoof diseases in animals (Christodoulopoulos, 2009; Mutebi et al., 2016).

1.2.2 Leucogentian violet

(a) Production process

Leucogentian violet is produced by the condensation of formaldehyde with *N*,*N*-dimethylaniline to form 4,4′-methylene-bis (*N*,*N*-dimethylaniline), which is reacted with additional *N*,*N*-dimethylaniline to yield the leuco base of gentian violet (Gessner & Mayer, 2000).

(b) Production volume

Leucogentian violet is manufactured in and/or imported to the European Economic Area in a volume of between 1 and 10 tonnes per annum (ECHA, 2020b). In 2020, leucogentian violet was available from 22 suppliers in China, 5 suppliers in the USA, 2 suppliers in India, and 1 supplier in Canada (Chemical Register, 2020b). [Data on quantities produced and used elsewhere in the world were not found by the Working Group.]

(c) Uses

Leucogentian violet is used as a precursor in the production of gentian violet dye (Gessner & Mayer, 2000). Leucogentian violet has been used as a chromogenic reagent for several analytical applications. Leucogentian violet is colourless and reacts quickly with oxidizers and free radicals to yield gentian violet, which is strongly coloured. The reaction can be readily observed by visualization or spectrophotometric analysis. Leucogentian violet is used in forensic analysis to enhance blood-impression evidence from fingerprints and footwear. Fixation with a 5-sulfosalicylic acid solution denatures proteins in the blood, allowing leucogentian violet to react with haem on the surface of the print. In the presence of hydrogen peroxide, haem catalyses the oxidation of leucogentian violet to gentian violet, producing the characteristic purple colour that results in enhanced print visualization (Spence & Asmussen, 2003; Bossers et al., 2011). Although other forensic dyes react with proteins and amino acids, the haem-sensitive reaction of leucogentian violet indicates the presence of blood. In analytical chemistry, the oxidation reaction of leucogentian violet to gentian violet has been used for sensitive spectrophotometric determination of hypochlorite, hydrogen peroxide, iodine/iodide, and metals (Borges & Reis, 2011). In a method for antimony determination, based on the reaction of antimony (III) with potassium iodate under acidic conditions to generate iodine, iodine oxidizes leucogentian violet to enable colorimetric detection (Tiwari et al., 2006). Leucogentian violet has also been used as a radiochromic indicator to enable the measurement of radiation exposure by dosimeters. Free radical production from gamma-radiation on a matrix can cause radiolytic oxidation of leucogentian violet, which generates a visible measure of radiation exposure (Dhevi et al., 2020).

Leucogentian violet is a metabolite resulting from the veterinary use of gentian violet for the treatment of fish and poultry. Residues of leucogentian violet may be found in fatty muscle and skin (WHO, 2014a).

1.3 Methods of detection and quantification

Representative methods for the detection and quantification of gentian violet and leucogentian violet are summarized in Table 1.1.

1.3.1 Air

No methods for the detection and quantification of gentian violet or leucogentian violet particulates in air were found.

1.3.2 Water

Gentian violet is measured in water for environmental monitoring and to determine the efficiency of physical, chemical, and biological methods to remove, decolourize, or degrade gentian violet in wastewater (Mani & Bharagava, 2016). Ultraviolet-visible absorbance techniques are commonly used to measure the reduction of the purple colour from highly concentrated wastewater samples, while liquid chromatography with spectroscopic or mass spectrometry detection is a more sensitive technique (Tkaczyk et al., 2020). For residue analysis in environmental water samples, pre-treatment procedures are required to concentrate gentian violet residues before analysis. Magnetic, ionic liquid, nanoparticle material, and microextraction techniques such as magnetic solid-phase extraction, dispersive liquid–liquid microextraction, micro-cloud point extraction, and monolithic fibre-based solid-phase microextraction have been used to isolate gentian violet residues from aqueous samples before analysis, with detection limits ranging from 0.03 to 5 µg/L (Šafařík

Table 1.1 Representative methods for the detection and quantification of gentian violet and leucogentian violet in various matrices

Sample matrix	Sample preparation	Analytical technique	Agent	LOD (unless otherwise stated)	Reference
Water					
Drinking- and river water	Magnetic SPE	Vis spectrophotometry	GV	0.5–1.0 µg/L	Šafařík & Šafaříková (2002)
Pond and effluent water	TC-IL-DLLME using 1-octyl-3-methylimidazolium hexafluorophosphate	HPLC-UV	GV	0.030 µg/L	Zhang et al. (2012)
Waste- and tap water	MCPE using Triton X-114	UV–vis spectrophotometry	GV	5.1 µg/L 17.6 µg/L (LOQ)	Ghasemi & Kaykhaii (2016)
Aquaculture water	Monolithic fibre SPME, evaporation, and reconstitution in methanol	HPLC-vis/FLD	GV LGV	0.14 µg/L 0.46 µg/L (LOQ) 0.013 µg/L 0.043 µg/L (LOQ)	Wang et al. (2015)
Soil					
River sediment and soil	Soxhlet extraction with 2-propanol	GC-MS	LGV	NR	Nelson & Hites (1980)
Food					
Dried tofu, chili sauce, seafood sauce, and tomato sauce	Extraction with MeOH/ACN, purification with d-SPE using PSA, GCB, alumina, and C_{18} filtration	LC-MS/MS	GV	0.03 µg/kg 0.09 µg/kg (LOQ)	Hu et al. (2020)
Beef, pork, chicken, egg, milk, flatfish, eel, and shrimp	Extraction with ACN/acetic acid, anhydrous sodium sulfate, purification with d-SPE using C_{18} and PSA filtration	LC-MS/MS	GV, LGV	2 µg/kg (LOQ)	Park et al. (2020)
Trout and shrimp	Extraction with HAH, ACN/ascorbic acid, anhydrous magnesium sulfate, and heated ultrasonic treatment	LC-MS/MS	GV LGV	0.15 µg/kg (CCα) 0.19 µg/kg (CCβ) 0.27 µg/kg (CCα) 0.43 µg/kg (CCβ)	Eich et al. (2020)
Trout, salmon, and prawns	Extraction with ACN, magnesium sulfate, filtration, oxidation with DDQ, evaporation, and reconstitution in ACN/ascorbic acid	LC-MS/MS	Sum of GV + LGV	0.02 µg/kg (CCα)	Dubreil et al. (2019)
Fish blood and extracts	Extraction with ACN, alumina-SPE, and TiO_2 nanoflake dispersion	SALDI-TOF-MS	GV	0.1 pg/mL	Gao et al. (2019)

Table 1.1 (continued)

Sample matrix	Sample preparation	Analytical technique	Agent	LOD (unless otherwise stated)	Reference
Trout, salmon, catfish, tilapia, shrimp, Arctic char, barramundi, eel, frog legs, hybrid striped bass, pompano, scallops, sea bream, smoked trout, dried shrimp, and highly processed canned eel and dace products; the canned products contained oil, salt, sugar, flavourings, spices, sauces, and/or preservatives	Extraction with HAH, ACN, magnesium sulfate, evaporation, reconstitution in ACN/ascorbic acid, and filtration	LC-MS/MS	GV LGV	< 0.5 µg/kg < 1.0 µg/kg (LOQ) 0.13 µg/kg (CCα) 0.17 µg/kg (CCβ) < 0.5 µg/kg < 1.0 µg/kg (LOQ) 0.42 µg/kg (CCα) 0.54 µg/kg (CCβ)	Andersen et al. (2018) Hurtaud-Pessel et al. (2011)
Trout, shrimp, humpback salmon, carp, mackerel, caviar, and crawfish	Extraction with ACN and water, and filtration	HPLC-HR-TOF-MS	GV LGV	0.01 µg/L 0.04 µg/L (LOQ) 0. 1 µg/L 0.4 µg/L (LOQ)	Amelin et al. (2017)
Eel	Extraction with ACN, sodium acetate, oxidation with DDQ, evaporation, McIlvaine buffer pH 6.5/ACN, CBA and SCX-SPE, evaporation, reconstitution in ammonium acetate buffer pH 4.5/ACN, and filtration	LC-MS/MS	Sum of GV + LGV	< 0.01 µg/kg 0.25 µg/kg (LOQ)	Reyns et al. (2014)
Salmon and shrimp	Extraction with citrate buffer/ACN, LLE with dichloromethane, SCX-SPE, filtration, post-column oxidation with PbO_2		GV LGV (detected as GV)	0.248 µg/kg (CCα) 0.335 µg/kg (CCβ) 0.860 µg/kg (CCα) 1.162 µg/kg (CCβ)	Ascari et al. (2012)
Silver carp, crucian carp, tilapia, mandarin fish, bream, and sea cucumber	Extraction with HAH/p-TSA/ammonium acetate/ACN, LLE with dichloromethane, diethylene glycol, ACN, evaporation, reconstitution in ACN, MCAX-SPE, evaporation, reconstitution in ammonium acetate/ACN/formic acid, and filtration	UPLC-MS/MS	GV LGV	0.15 µg/kg 0.50 µg/kg (LOQ) 0.15 µg/kg 0.50 µg/kg (LOQ)	Xu et al. (2012)

Table 1.1 (continued)

Sample matrix	Sample preparation	Analytical technique	Agent	LOD (unless otherwise stated)	Reference
Salmon	Extraction with ammonium acetate buffer pH 4.5, ACN, d-SPE with alumina, LLE with dichloromethane, formic acid, oxidation with DDQ, and SCX-SPE	LC-MS/MS	Sum of GV + LGV	1.4 µg/kg (CCα) 2.4 µg/kg (CCβ)	Tarbin et al. (2008)
Biospecimens					
Human urine	SPE	HPLC-ECD	GV	0.5 µg/L	Sagar et al. (1995)

ACN, acetonitrile; CBA, cation exchange cartridges; CCα, decision limit: the concentration level at which there is probability α (usually defined as 1% for non-authorized substances) that a blank sample will give a signal at this level or higher; CCβ, detection capability: the concentration level at which there is a probability β (usually defined as 5%) that the method will give a result lower than CCα; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; d-SPE, dispersive solid-phase extraction; ECD, electrochemical detection; GC, gas chromatography; GCB, graphitized carbon black; GV, gentian violet; HAH, hydroxylamine hydrochloride; HPLC, high-performance liquid chromatography; HR-TOF, high-resolution quadrupole time-of-flight; LC, liquid chromatography; LGV, leucogentian violet; LLE, liquid–liquid extraction; LOD, limit of detection; LOQ, limit of quantification; MCAX, mixed-mode cation exchange; MCPE, micro-cloud point extraction; MeOH, methanol; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NR, not reported; PbO_2, lead (II) oxide; PSA, primary secondary amine; *p*-TSA, *para*-toluenesulfonic acid; SALDI-TOF, surface-assisted laser desorption/ionization time-of-flight; SCX, strong cation exchange; SPE, solid-phase extraction; SPME, solid-phase microextraction; TC-IL-DLLME, temperature-controlled ionic liquid dispersive liquid–liquid microextraction; TiO_2, titanium dioxide; UPLC, ultra-performance liquid chromatography; UV, ultraviolet; vis, visible light; vis/FLD, visible light and fluorescence detection.

& Šafaříková, 2002; Zhang et al., 2012; Wang et al., 2015; Ghasemi & Kaykhaii, 2016; Moradi Shahrebabak et al., 2020).

1.3.3 Soil

Leucogentian violet has been identified in soil near waste discharged from a dye-manufacturing plant by means of Soxhlet extraction with 2-propanol and analysis by gas chromatography-tandem mass spectrometry (Nelson & Hites, 1980).

1.3.4 Food, beverages, and consumer products

Gentian violet is not permitted for use as a food additive, but numerous methods have been developed to determine residues of gentian violet and its metabolite, leucogentian violet, in animal products as a result of veterinary treatment with gentian violet (WHO, 2014a; Verdon & Andersen, 2017). In gentian violet-treated fish, the major metabolite (leucogentian violet) has a longer residence time (> 79 days) than gentian violet (~5 days) in fish (Thompson et al., 1999). Thus, leucogentian violet is the marker residue used to monitor gentian violet use in aquaculture, and seafood analysis methods must assess both compounds. Many early methods of residue analysis were based on the extraction of muscle with an acidic buffer and acetonitrile, liquid–liquid partitioning, and solid-phase clean-up with alumina, followed by high-performance liquid chromatography (HPLC) separation (Roybal et al., 1990). Several approaches have been used to enable the detection of both the chromatic dye and the colourless leuco base, including electrochemical detection, post-column oxidation of leucogentian violet with lead oxide (Ascari et al., 2012), and simultaneous visible (gentian violet absorbs at 588 nm) and fluorescence (leucogentian violet excitation at 265 nm with emission at 360 nm) detection (Verdon & Andersen, 2017). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have largely replaced HPLC to meet low-concentration regulatory monitoring levels (e.g. 0.5 µg/kg) for direct quantification of the dye and leuco ions (Hurtaud-Pessel et al., 2011; Xu et al., 2012; Andersen et al., 2018; Eich et al., 2020). Some multiresidue LC-MS/MS methods for the detection of therapeutic dyes in seafood include the oxidation of leuco compounds with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone to ensure that dye metabolites are also detected (Tarbin et al., 2008; Andersen et al., 2009; Reyns et al., 2014; Dubreil et al., 2019). A method has been developed to extract gentian violet and leucogentian violet from zebrafish using a solid-phase microextraction probe, which detects residues via direct ionization mass spectrometry from the probe (Xiao et al., 2020). [The Working Group noted that the novel method employed in the study of zebrafish (which are not typically eaten) could have applicability in fish species that are consumed by humans.] Additional multidye LC-MS/MS methods that include sensitive quantification of gentian violet (0.09–2 µg/kg) have been applied to the analysis of foods such as dried tofu and sauces (Hu et al., 2020), and beef, chicken, pork, eggs, and milk (Park et al., 2020). High-resolution mass spectrometry has also been used for the detection and quantification of gentian violet and leucogentian violet (Amelin et al., 2017).

Surface-enhanced Raman scattering and direct mass spectrometry techniques have also been used to detect gentian violet. Silver nanoparticle films and pastes have been used to detect gentian violet on the surface of fish skin and in ballpoint pen ink (Alyami et al., 2019; Saviello et al., 2019). A surface-assisted laser desorption/ionization mass spectrometry method has been used to analyse gentian violet in printed supermarket receipts (Gao et al., 2019).

1.3.5 Biological specimens

Methods for the detection and quantification of gentian violet and leucogentian violet in human biological specimens are similar to those used for food (as described in Section 1.3.4). Gentian violet and leucogentian violet have been determined in human urine via extraction of neutralized urine with dichloromethane, extract clean-up with sodium sulfate, and analysis by HPLC with absorbance or electrochemical detection (Sagar et al., 1995). [The Working Group noted that the methods used for gentian violet and leucogentian violet detection in fish described in Section 1.3.4 could be useful for analysing material from humans or experimental animals. For biological specimen analysis, it might be more important to monitor N-demethylated and/or *N*-oxide metabolites of gentian violet and leucogentian violet.]

1.4 Occurrence and exposure

1.4.1 Environmental occurrence

Gentian violet is not known to occur naturally in the environment. Gentian violet and leucogentian violet production and their use (e.g. during the production of ink cartridges and coloured paper, and during the recycling of printed paper) may result in the release of these compounds into the environment via streams of both industrial and municipal wastewater (Health Canada, 2020; Tkaczyk et al., 2020).

When released into the environment, gentian violet exists in cationic form. Considering its physicochemical properties, gentian violet exists only in the particulate phase in the atmosphere. [The Working Group also noted that the water solubility of gentian violet is several orders of magnitude higher than that of leucogentian violet and that the octanol/water partition coefficient of gentian violet is one order of magnitude higher, which has implications for its fate in the environment.] Particulate-phase gentian violet is removed from the atmosphere by wet and dry deposition and may be susceptible to direct photolysis by sunlight. Gentian violet is expected to be immobile if released into soil. Soils containing organic carbon and clay will adsorb gentian violet's cationic form more strongly than its neutral counterpart. Volatilization from moist soil is not expected. According to the transformation rates observed during a river die-away test, biodegradation may be an important environmental process in soil and water. If released into water, gentian violet is expected to adsorb on suspended solids and sediment, and the non-adsorbed fraction will exist almost entirely in the cationic form; therefore, volatilization from water is not expected. Gentian violet is not expected to undergo hydrolysis in the environment (NCBI, 2013).

Leucogentian violet was detected in a soil sample taken near a bank of the Buffalo River, New York, close to a dyestuff-manufacturing plant (Nelson & Hites, 1980). Theoretical estimations of concentrations of non-sulfonated triarylmethane dyes in surface water (also representing drinking-water) were calculated for three industrial sources in Canada based on the maximum production capacities of these industries: 3.2×10^{-4} mg/L from the paper-dyeing industry, 9.5×10^{-4} mg/L from the de-inking industry, and 2.1×10^{-4} mg/L from the general formulation industry. These conservative estimates were made for gentian violet, malachite green, and two other triarylmethane dyes collectively, assuming that any one of the four dyes could be substituted for another (Health Canada, 2020). In the National Water Pollution Control and Treatment Project in Dong Lin, China, gentian violet concentrations of 0.87 and 0.049 µg/L were found in the water from turtle farming ponds and effluent environmental water, respectively (Zhang et al., 2012). Gentian violet absorbs light at an ultraviolet maximum of 590 nm with potential for direct photolysis. In water, the

photoreaction is reported to give *para*-dimethylamino phenol and 4,4′-bis(dimethylamino) benzophenone, the leuco and demethylated derivatives of gentian violet. The bioconcentration in aquatic organisms is low, as suggested by the estimated bioconcentration factor of 3 L/kg in fish (NCBI, 2013), but such models may not be appropriate for triarylmethane dyes because of their cationic nature. For these triarylmethanes, partitioning to proteins in the cell membranes is more likely to occur than partitioning to lipids (Health Canada, 2020).

A study was performed to analyse the presence of 16 dyes, which included triarylmethanes and their metabolites such as gentian violet and leucogentian violet, in wild fish in Belgium. Muscle samples were analysed from individual yellow-phased European eels (*Anguilla anguilla*) from 91 locations in rivers, canals, and lakes sampled between 2000 and 2009. Gentian violet and leucogentian violet were detected in samples from 58.2% and 50.5% of the locations, respectively. The concentrations of gentian violet and leucogentian violet ranged between 0.12 and 2.60 µg/kg (Belpaire et al., 2015). In an earlier study conducted in Germany, gentian violet and leucogentian violet were found in tissue samples from wild eels caught in seven out of eight receiving waters of effluents from municipal sewage treatment plants. The concentrations of gentian violet and leucogentian violet ranged from 0.06 to 6.71 µg/kg (Schuetze et al., 2008).

1.4.2 Occurrence in food and feed

Gentian violet is used in veterinary medicine and in the aquaculture industry for the control of ectoparasites, and fungal and bacterial infections. Residues of both gentian violet and leucogentian violet may be present in muscle and skin after gentian violet treatment. Although gentian violet metabolizes within days of treatment, leucogentian violet persists in fish muscle and skin for months and is considered to be the marker residue (Thompson et al., 1999). [The Working Group noted that in the reports described below, the methods either detected gentian violet and leucogentian violet separately, or detected total residues as the sum of gentian violet and leucogentian violet after leucogentian violet had been oxidized to gentian violet.]

According to the European Food Safety Authority reports published between 2015 and 2020, few Member States (one to four) reported one or two samples that were non-compliant for the presence of gentian violet and leucogentian violet in their national veterinary drug residue control plan (EFSA, 2015, 2016, 2017, 2018, 2019, 2020). In the European Rapid Alert System for Food and Feed, very few notifications of non-compliant samples associated with imports or trade between Member States have been reported. Since 2005, 15 notifications of gentian violet or leucogentian violet residue violations have been made by EU Member States in eel, salmon, tilapia, rainbow trout, catfish, pangasius, and sturgeon (caviar). Residue concentrations have typically ranged from 0.8 to 6.6 µg/kg, although two high-concentration (41.1 and 654.6 µg/kg) samples were reported for eel from Indonesia in 2006 (European Commission, 2020).

In a study of processed fish and shrimp samples in Korean local markets, gentian violet was detected (168.4 µg/kg) in 1 of 67 eel samples tested. It was not detected in the other 186 processed fish and shrimp samples, which originated from the Republic of Korea, China, Thailand, Viet Nam, Norway, Peru, and the Russian Federation, or were of unknown origin (Lee et al., 2010). Among fish obtained from a local market in China, 7.15 µg/kg of gentian violet was detected in tilapia; none was detected in carp, sea cucumber, or seashell (Xu et al., 2012). Among 20 salmon and shrimp samples purchased from different markets in China, 1.2 µg/kg of gentian violet and 2.5 µg/kg of leucogentian violet were detected in one salmon

muscle sample (Tao et al., 2011). Leucogentian violet (0.6–1.0 µg/kg) was detected in 5 out of 208 samples of rainbow trout obtained from local fish retailers and supermarkets in Turkey (Kaplan et al., 2014). In the Russian Federation, 5.3 µg/kg of gentian violet was detected in black caviar (Amelin et al., 2017). Gentian violet and leucogentian violet residues have also been reported for samples tested in the USA, Canada, and Jordan (Table 1.2; WHO, 2014a; Gammoh et al., 2019). In the EU and USA, respectively, 3% and 6% of reported veterinary drug violations detected in finfish in 2001–2008 and 2001–2006, respectively, were due to the detection of gentian violet. The concentrations detected in the EU and the USA did not differ (Love et al., 2011).

In a screening study of 19 commercially available processed animal products (salmon feed ingredients) from central Europe, leucogentian violet was detected in one poultry blood-meal sample (Nácher-Mestre et al., 2016).

1.4.3 Occupational exposure

Occupational exposure to gentian violet is expected to occur via dermal contact during paper dyeing, via inhalation of dust or aerosols produced during the formulation of dye or ink, or during the filling of containers such as ink cartridges and ballpoint pens (ECHA, 2012). [The Working Group noted that occupational exposure to gentian violet and leucogentian violet may occur through dermal contact and inhalation at workplaces where the compounds are produced or applied (see Sections 1.1.2 and 1.2.2).] In a survey conducted in the USA in 1981–83, 75 632 people were estimated to be potentially occupationally exposed to gentian violet: 69% of them working in health services, 12% in printing and publishing, and 8% in agricultural services (NIOSH, 2017). [The Working Group noted that it is unclear whether these percentages reflect modern exposure patterns, given the age of the study.]

1.4.4 Exposure in the general population

The predominant source of exposure to dye substances in the triarylmethanes group is from the use of products that contain them that are available to consumers (Health Canada, 2020). Exposure of the general population can potentially occur during the use of the consumer products described in Section 1.1.2, such as ballpoint and marker pens (orally by sucking or via dermal contact), topical treatments for animals (inhalation or dermal), coloured paper, hair dye, aquarium fish treatments, or through the consumption of contaminated drinking-water or residue-containing fish (Table 1.2). A screening assessment performed by Health Canada suggested exposure via drinking-water to be the main route of exposure to gentian violet. A potential dose of 0.0001 mg/kg body weight (bw) per day was estimated for the Canadian general population on the basis of predicted surface water concentrations as a result of environmental release by the paper de-inking industry. Other exposure scenarios considered, but not included in the estimation because of lower estimated exposures, were surface water due to industrial release from paper dyeing in paper mills and production facilities, and consumer "down-the-drain" releases, consumption via food, and the use of consumer products such as paper products, mixtures, or manufactured items in which gentian violet is used as a pigment (Health Canada, 2020).

[The Working Group noted that despite the multitude of sources, no quantitative exposure data were available.]

1.5 Regulations and guidelines

1.5.1 Exposure limits and guidelines

Gentian violet is listed by the European Chemicals Agency as a carcinogen (Category 2) and as a carcinogen (Category 1B) when the

Table 1.2 Detection and quantification of gentian violet and leucogentian violet in aquaculture products available on the international market[a]

Country reported	Country of origin	Agent	Year	Sample type	No. of samples tested	No. of positive samples (%)	Concentration (µg/kg)		Reference
							Mean ± SD	Range	
Canada	–	GV and LGV	2008–2009	Tilapia, salmon, and shrimp	135	6 (4.4)	2.48 ± 2.32	0.64–5.60	WHO (2014a)
		GV and LGV	2009–2010	NA	484	0	NA	NA	
		GV and LGV	2010–2011	Tilapia, perch, shrimp, milkfish, and catfish	542	11 (2.0)	1.92 ± 1.69	0.50–4.30	
		GV and LGV	2011–2012	Bass and prawn	396	2 (0.5)	2.23 ± 2.02	0.80–3.65	
		GV and LGV	2012–2013	Perch and dried fish maw	269	3 (1.1)	3.06 ± 2.07	0.98–5.12	
USA	–	GV and LGV	2004	NA	622	0	NA	NA	WHO (2014a)
		GV and LGV	2005	NA	536	0	NA	NA	
		GV and LGV	2006	NA	588	0	NA	NA	
		GV and LGV	2007	Eel, catfish, and shrimp	686	3+ (0.4)[b]	NR	2.5–26.9	
Jordan	Viet Nam	GV		*Pangasius*	27	17 (62)	11.7	0.362–41.3	Gammoh et al. (2019)
		LGV		*Pangasius*	27	5 (18)	5.26	0.178–10.58	
	United Arab Emirates	GV		*Pangasius*	27	8 (29)	4.4	0.945–10.6	
		LGV		*Pangasius*	27	NA	NA	NA	
	China	GV		Tilapia	27	11 (40)	4.6	1.24–9.48	
		LGV		Tilapia	27	2 (7)	2.1	1.29–2.81	
	Argentina	GV		Argentine hake	20	NA	NA	NA	
		LGV		Argentine hake	20	NA	NA	NA	
	USA	GV		Pacific hake	20	NA	NA	NA	
		LGV		Pacific hake	20	NA	NA	NA	
	All countries above, reported by Jordan	GV		*Pangasius*, tilapia, Argentine hake, and Pacific hake	121	36 (30)	6.9	0.362–41.3	
		LGV		*Pangasius*, tilapia, Argentine hake, and Pacific hake	121	7 (5.7)	3.2	0.178–10.58	

Table 1.2 (continued)

Country reported	Country of origin	Agent	Year	Sample type	No. of samples tested	No. of positive samples (%)	Concentration (μg/kg)		Reference
							Mean ± SD	Range	
Republic of Korea	China Republic of Korea, China, Thailand, Viet Nam, Norway, Peru, the Russian Federation	Sum of GV + LGV		Eel Fish and shrimp	7 246	1 (14) 0	168.4 NA	NA NA	Lee et al. (2010)
China	China	GV GV		Tilapia Carp, sea cucumber, and seashell	NR NR	NR 0	7.15 NA	NR NA	Xu et al. (2012)
China	China	GV LGV		Salmon and shrimp	20	1 (5) 1 (5)	1.2 2.5	NA NA	Tao et al. (2011)
Turkey	Turkey	LGV		Rainbow trout	208	5 (2.4)	[0.70]	0.52–1.0	Kaplan et al. (2014)
Russian Federation	Russian Federation?	GV		Sturgeon caviar	1	1 (NA)	5.3	NA	Amelin et al. (2017)

GV, gentian violet; LGV, leucogentian violet; NA, not applicable; NR, not reported; SD, standard deviation.

[a] Monitoring of gentian violet and leucogentian violet by Canada and the USA, in frozen fish imported to Jordan, and in aquaculture products sold in local markets in China, the Republic of Korea, Turkey, and the Russian Federation.

[b] Probably an underestimate.

Michler's ketone or Michler's base impurity is present at 0.1% or more (ECHA, 2012). It is classified as a substance of very high concern (ECHA, 2012). Gentian violet is very toxic to aquatic life (acute H400 and chronic H410), is harmful if swallowed (H302), causes serious eye damage (H318), and is suspected of causing cancer (H350) (ECHA, 2020a).

The Joint Food and Agriculture Organization of the United Nations/WHO Expert Committee on Food Additives (JEFCA) concluded that there is no acceptable daily intake or maximum residue limit for gentian violet and its marker leucogentian violet (WHO, 2014a). Gentian violet is not authorized for use as a veterinary drug in the Australia, Brazil, Canada, Chile, the EU, New Zealand, or the UK, and there is zero tolerance for residues of gentian violet in food for human consumption (Verdon & Andersen, 2017; Health Canada, 2019). In the USA, gentian violet is not permitted for use in animal feeds or as a veterinary drug for food-producing animals (US FDA, 2007). Gentian violet and leucogentian violet are not permitted for use as food additives or in food packaging in the USA (US FDA 2020, 2021). In Canada, gentian violet is not permitted for use in animal feeds or in aquaculture production (Health Canada, 2018).

In food products derived from animals where gentian violet is prohibited for use, there is zero tolerance for residues of gentian violet and/or its metabolite leucogentian violet, which is the marker residue that indicates the use of gentian violet (WHO, 2014a). Reference points for action range from 0.5 to 2.0 µg/kg, as determined by the detection capabilities of the analytical methods used in national and international residue monitoring programmes for each compound, or for the sum of gentian violet and leucogentian violet residues (Verdon & Andersen, 2017).

Gentian violet is not permitted for use as a hair dye in the European Economic Area (European Commission, 2009), and it is not approved for any cosmetic use in Canada, New Zealand, or Singapore (Health Canada, 2018; NZ EPA, 2019; HSA, 2020). United States Food and Drug Administration regulations require that hair dyes containing gentian violet are accompanied by a cautionary statement for skin and eye irritation, with instructions to perform a skin patch test before use (Diamante et al., 2009).

No stand-alone regulations were found for leucogentian violet.

1.5.2 Reference values for biological monitoring of exposure

No reference values for biological monitoring of gentian violet or leucogentian violet exposure were found.

2. Cancer in Humans

No data were available to the Working Group.

3. Cancer in Experimental Animals

3.1 Gentian violet

See Table 3.1.

3.1.1 Mouse

Oral administration (feed)

In a study of chronic toxicity and carcinogenicity that complied with Good Laboratory Practice (GLP) (NCTR, 1984; Littlefield et al., 1985), a total of 720 male and 720 female B6C3F$_1$ mice (age, approximately 4–5 weeks) were given feed containing gentian violet (purity, 99%; methyl violet, 1%) at a concentration of 0, 100, 300, or 600 ppm [approximately equivalent to 0, 12.5, 33.9, and 66.1 mg/kg bw per day for males, and 0, 14.3, 37.5, and 71.4 mg/kg bw per day for females] for the control group and the groups at the lowest, intermediate, and highest

Table 3.1 Studies of carcinogenicity with gentian violet in experimental animals

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (M) ~4–5 wk 24 mo Littlefield et al. (1985)	Oral Purity, 99% (impurity, 1% methyl violet) Feed 0, 100, 300, 600 ppm 192, 96, 96, 96 167, 83, 77, 74	*Liver* Hepatocellular adenoma 17/183, 14/92, 20/93*, 37/93**	[$P < 0.001$, Cochran–Armitage trend test; *$P < 0.01$, **$P < 0.001$, one-tailed Fisher exact test]	Principal strengths: complied with GLP; used males and females; adequate duration of exposure and observation; high number of mice per group Other comments: the incidence of hepatocellular adenoma or carcinoma (combined) was not reported
		Hepatocellular carcinoma 27/183, 15/92, 17/93, 33/93*	$P < 0.001$, trend test; *$P < 0.01$, one-tailed Fisher exact test	
		Harderian gland: adenoma 7/187, 7/92, 10/94*, 9/89**	*$P < 0.05$, one-tailed Fisher exact test; [**$P = 0.0362$, one-tailed Fisher exact test]; [NS], Cochran–Armitage trend test	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (F) ~4–5 wk 24 mo Littlefield et al. (1985)	Oral Purity, 99% (impurity, 1% methyl violet) Feed 0, 100, 300, 600 ppm 192, 96, 96, 96 167, 69, 70, 35	*Liver* Hepatocellular adenoma 8/185, 8/93, 36/93*, 20/95**	[$P < 0.001$, Cochran–Armitage trend test; *$P < 0.01$, **$P < 0.001$; one-tailed Fisher exact test]	Principal strengths: complied with GLP; used males and females; adequate duration of exposure and observation; high number of mice per group Other comments: the incidence of hepatocellular adenoma or carcinoma (combined) was not reported
		Hepatocellular carcinoma 7/185, 5/93, 30/93*, 73/95*	[$P < 0.001$, Cochran–Armitage trend test, trend test; *$P < 0.001$, one-tailed Fisher exact test]	
		Harderian gland: adenoma 8/186, 11/93*, 18/89**, 15/94***	$P = 0.001$, Cochran–Armitage trend test; *$P < 0.05$, **$P < 0.001$, ***$P < 0.005$, one-tailed Fisher exact test	
		Bladder: reticulum cell sarcoma type A [histiocytic sarcoma] 0/188, 2/92, 3/89*, 5/91**	[$P < 0.005$, Cochran–Armitage trend test; *$P < 0.05$, **$P < 0.01$; one-tailed Fisher exact test]	
		Ovaries: reticulum cell sarcoma type A [histiocytic sarcoma] 0/178, 1/90, 3/89*, 5/89**	[$P = 0.009$, Cochran–Armitage trend test; *$P = 0.036$, **$P = 0.04$; one-tailed Fisher exact test]	
		Uterus: reticulum cell sarcoma type A [histiocytic sarcoma] 0/188, 2/95, 6/90*, 12/93**	[$P < 0.001$, Cochran–Armitage trend test; *$P < 0.01$, **$P < 0.001$; one-tailed Fisher exact test]	
		Vagina: reticulum cell sarcoma type A [histiocytic sarcoma] 1/182, 1/90, 4/88*, 8/87**	[$P = 0.001$, Cochran–Armitage trend test; *$P = 0.04$, **$P < 0.001$; one-tailed Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (M) ~4–5 wk 18 mo Littlefield et al. (1985)	Oral Purity, 99% (impurity, 1% methyl violet) Feed 0, 100, 300, 600 ppm 48, 24, 24, 24 NR	*Liver* Hepatocellular adenoma 3/48, 0/24, 2/24, 2/22 Hepatocellular carcinoma 5/48, 1/24, 2/24, 2/22 *Harderian gland*: adenoma 2/47, 2/24, 2/23, 0/21	[NS] [NS] [NS]	Principal strengths: complied with GLP; used males and females Principal limitations: small number of mice per treated group Other comments: the incidence of hepatocellular adenoma or carcinoma (combined) was not reported
Full carcinogenicity Mouse, B6C3F$_1$ (F) ~4–5 wk 18 mo Littlefield et al. (1985)	Oral Purity, 99% (impurity, 1% methyl violet) Feed 0, 100, 300, 600 ppm 48, 24, 24, 24 NR	*Liver* Hepatocellular adenoma 3/47, 0/22, 3/24, 8/24* Hepatocellular carcinoma 1/47, 0/22, 1/24, 3/24 *Harderian gland*: adenoma 2/46, 2/21, 3/23, 1/23 *Uterus*: reticulum cell sarcoma type A [histiocytic sarcoma] 0/47, 0/22, 1/24, 1/24 *Bladder*: reticulum cell sarcoma type A [histiocytic sarcoma] 0/47, 1/22, 1/24, 0/23 *Vagina*: reticulum cell sarcoma type A [histiocytic sarcoma] 0/46, 0/22, 1/23, 0/22	[P = 0.002, Cochran–Armitage trend test; *P = 0.005, one-tailed Fisher exact test] [NS] [NS] [NS] [NS] [NS]	Principal strengths: complied with GLP; used males and females Principal limitations: small number of mice per treated group Other comments: the incidence of hepatocellular adenoma or carcinoma (combined) was not reported

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (M) ~4–5 wk 12 mo NCTR (1984)	Oral Purity, 99% (impurity, 1% methyl violet) Feed 0, 100, 300, 600 ppm 48, 24, 24, 24 NR	*Liver*: hepatocellular adenoma 0/48, 2/24, 0/24, 0/24	[NS]	Principal strengths: complied with GLP; used males and females Principal limitations: small number of mice per treated group Other comments: the incidence of hepatocellular adenoma or carcinoma (combined) was not reported
Full carcinogenicity Mouse, B6C3F$_1$ (F) ~4–5 wk 12 mo NCTR (1984)	Oral Purity, 99% (impurity, 1% methyl violet) Feed 0, 100, 300, 600 ppm 48, 24, 24, 24 NR	*Harderian gland*: adenoma 2/48, 0/24, 1/24, 0/24 *Vagina*: reticulum cell sarcoma type A [histiocytic sarcoma] 0/45, 1/23, 0/24, 0/23	[NS] [NS]	Principal strengths: complied with GLP; used males and females Principal limitations: small number of mice per treated group Other comments: the incidence of hepatocellular adenoma or carcinoma (combined) was not reported

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Rat, F344 (M) NR (weanling) 24 mo NCTR (1988)	Transplacental and perinatal exposure, followed by oral administration (feed) Purity, 99% (impurity, 1% methyl violet) Feed 0, 100, 300, 600 ppm 180, 90, 90, 90 121, 60, 47, 55	*Liver*: hepatocellular adenoma 1/179, 2/90, 3/88*, 4/89*	$P < 0.01$, Peto trend test; *$P < 0.01$, Peto test and Bonferroni correction One rat at 100 ppm had a hepatocellular carcinoma	Principal strengths: complied with GLP; used males and females; adequate duration of exposure and observation; high number of rats per group
		Thyroid gland Follicular cell adenoma 1/163, 0/84, 0/74, 2/79	[NS]	
		Follicular cell adenocarcinoma 1/163, 4/84*, 2/74, 5/79**	$P < 0.01$, Peto trend test; *$P < 0.05$, **$P < 0.01$, Peto test and Bonferroni correction	
		Follicular cell adenoma or adenocarcinoma (combined) 2/163, 4/84, 2/74, 7/79*	[$P < 0.05$, Cochran–Armitage trend test; *$P < 0.01$, one-tailed Fisher exact test]	
		Testis and epididymis: mesothelioma 3%, 2%, 6%, 9%	NR, incidence reported only as percentage	
		Multiple organs: mononuclear cell leukaemia 104/180, 66/90, 69/90, 51/90	NS	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Rat, F344 (F) NR (weanling) 24 mo NCTR (1988)	Transplacental and perinatal exposure, followed by oral administration (feed) Purity, 99% (impurity, 1% methyl violet) Feed 0, 100, 300, 600 ppm 180, 90, 90, 90 121, 56, 36, 31	*Liver*: hepatocellular adenoma 0/170, 1/90, 2/83, 1/87 *Thyroid gland* Follicular cell adenoma 1/159, 2/83, 3/76, 3/77 Follicular cell adenocarcinoma 1/159, 1/83, 4/76*, 6/77** Follicular cell adenoma or adenocarcinoma (combined) 2/159, 3/82, 7/76*, 9/77** *Multiple organs*: mononuclear cell leukaemia 77/171, 38/90, 45/87, 40/87 *Clitoral gland*: adenoma or adenocarcinoma (combined) 12%, 6%, 18%, 33%	NS [NS] $P < 0.01$, Peto trend test; *$P < 0.05$, **$P < 0.01$, Peto test and Bonferroni correction [$P < 0.01$, Cochran–Armitage trend test; *$P < 0.01$, **$P < 0.001$, one-tailed Fisher exact test] NS NR, incidences reported only as percentages	Principal strengths: complied with GLP; used males and females; adequate duration of exposure and observation; high number of rats per group

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Rat, F344 (M) NR (weanling) 18 mo Littlefield et al. (1989)	Transplacental and perinatal exposure, followed by oral administration (feed) Purity, 99% (impurity, 1% methyl violet) Feed 0, 100, 300, 600 ppm 15, 15, 15, 15 NR	*Liver*: hepatocellular adenoma 0/15, 1/15, 0/15, 0/14 *Thyroid gland* Follicular cell adenoma 0/15, 0/15, 1/15, 1/15 Follicular cell adenoma or adenocarcinoma (combined) 0/15, 0/15, 1/15, 1/15 *Testis and epididymis*: malignant mesothelioma 0%, 0%, 13%, 13%	[NS] [NS] [NS] NR, incidences reported only as percentages	Principal strengths: complied with GLP; used males and females Principal limitations: small number of rats per group
Full carcinogenicity Rat, F344 (F) NR (weanling) 18 mo Littlefield et al. (1989)	Transplacental and perinatal exposure, followed by oral administration (feed) Purity, 99% (impurity, 1% methyl violet) Feed 0, 100, 300, 600 ppm 15, 15, 15, 15 NR	*Thyroid gland* Follicular cell adenocarcinoma 0/15, 1/11, 0/10, 0/14 Follicular cell adenoma or adenocarcinoma (combined) 0/15, 1/11, 0/10, 0/14 *Multiple organs*: mononuclear cell leukaemia 0/15, 2/11, 2/10, 6/14*	 [NS] [NS] [$P < 0.05$, Cochran–Armitage trend test; *$P < 0.01$, one-tailed Fisher exact test]	Principal strengths: complied with GLP; used males and females Principal limitations: small number of rats per group

F, female; GLP, Good Laboratory Practice; M, male; mo, month; NR, not reported; NS, not significant; ppm, parts per million; wk, week.

dose, respectively, for up to 24 months. The feed containing gentian violet was certified to be within 10% of the target dose. For the mice treated for 24 months, there were 192 males and 192 females in the control group and 96 males and 96 females in each group treated with gentian violet. For the mice treated for 12 or 18 months, there were 48 males and 48 females in the control group and 24 males and 24 females in each group treated with gentian violet. Mortality was very low until approximately 450 days (15 months), after which there was a significant positive dose-related trend in males (P = 0.01288, Cochran–Armitage test) and females (P = 0.00005, Cochran–Armitage test), with mortality being significantly higher in all treated groups of females compared with controls. At study termination, survival was 167/192, 83/96, 77/96, and 74/96 in males, and 167/192, 69/96, 70/96, and 35/96 in females, for the control group and the groups at the lowest, intermediate, and highest dose, respectively. Treatment with gentian violet did not influence the terminal body weights of males or females. Complete necropsies and histopathological examinations were performed.

In male mice at 24 months, there was a significant positive trend in the incidence of hepatocellular adenoma [$P < 0.001$, Cochran–Armitage trend test] and of hepatocellular carcinoma ($P < 0.001$, trend test), with a significant increase in the incidence of hepatocellular adenoma at the intermediate and highest dose [$P < 0.01$ and $P < 0.001$, respectively, one-tailed Fisher exact test], and of hepatocellular carcinoma at the highest dose [$P < 0.01$, one-tailed Fisher exact test]. The incidence of Harderian gland adenoma was also significantly increased at the intermediate and highest dose ($P < 0.05$ and [$P = 0.0362$], respectively, one-tailed Fisher exact test). At 12 or 18 months, no treatment-associated neoplasms were reported in males.

In female mice at 24 months, there was a significant positive trend in the incidence of hepatocellular adenoma and of hepatocellular carcinoma (both $P < 0.001$, Cochran–Armitage trend test), with a significant increase in the incidence of hepatocellular adenoma [$P < 0.01$ and $P < 0.001$, one-tailed Fisher exact test] and of hepatocellular carcinoma (both $P < 0.001$, one-tailed Fisher exact test) at the intermediate and highest dose, respectively, when compared with controls. Treatment with gentian violet caused a significant positive trend in the incidence of Harderian gland adenoma (P = 0.001, Cochran–Armitage trend test), with the incidence being significantly higher at the lowest, intermediate, and highest dose [$P < 0.05$, $P < 0.001$, and $P < 0.005$, respectively, one-tailed Fisher exact test] than in controls. Significant positive trends in the incidence of type A reticulum cell sarcoma [histiocytic sarcoma] were reported for the urinary bladder, ovaries, uterus, and vagina [$P < 0.0005$, $P = 0.009$, $P < 0.001$, $P = 0.001$, respectively, Cochran–Armitage trend test], with a significant increase in incidence (urinary bladder, $P < 0.05$ and $P < 0.01$; ovaries, $P = 0.036$ and $P = 0.04$; uterus, $P < 0.01$ and $P < 0.001$; and vagina, $P = 0.04$ and $P < 0.001$, Fisher exact test) at the intermediate and highest dose, respectively. At 18 months, a significant positive trend in the incidence of hepatocellular adenoma (P = 0.002, Cochran–Armitage trend test) was observed, with the increase being significant (P = 0.005, one-tailed Fisher exact test) at the highest dose. At 12 months, treatment with gentian violet did not cause a significant increase in the incidence of tumours in female mice.

Regarding non-neoplastic lesions observed at 24 months, exposure to gentian violet caused a significant positive trend and an increase in the incidence of erythropoiesis in the spleen and atrophy of the ovaries in females treated with gentian violet compared with controls. [The Working Group noted that this was a well-conducted study that complied with GLP, males and females were used, the duration of exposure and

observation was adequate, and a high number of mice per group was used.]

3.1.2 Rat

(a) Oral administration

In a study in rats [age and strain not reported], oral administration [regimen not reported] of 4:4′:4′′-hexamethyltriaminotriphenylmethane [gentian violet, purity not reported] for more than 300 days caused gastric papilloma and adenomatous proliferation in the hepatic tissue (Kinosita, 1940). [The Working Group noted that the study lacked details on study design and primary data and was considered inadequate for the evaluation of the carcinogenicity of gentian violet in experimental animals.]

(b) Transplacental and perinatal exposure, followed by oral administration (feed)

In a study of chronic toxicity and carcinogenicity that complied with GLP (NCTR, 1988; Littlefield et al., 1989), groups of male and female Fischer 344 rats (F_0 generation) (180 controls and 90 treated rats per group) were given feed containing gentian violet (purity, 99%; methyl violet, 1%) at a concentration of 0, 100, 300, or 600 ppm, for the control group, and the groups at the lowest, intermediate, and highest dose, respectively, for at least 80 days. While still receiving treated feed, female rats were mated with males that were receiving the same doses of gentian violet. Two offspring (F_1 generation) of each sex were randomly selected from each litter and three rats allocated per cage as weanlings [age, not reported] to the study of chronic toxicity and carcinogenicity. The F_1 rats were exposed to the same doses as their respective F_0 parents for up to 24 months. [These dose levels were approximately equivalent to 0, 4.3, 11.4, and 22.9 mg/kg bw per day for male F_1 rats, and 0, 5.7, 14.3, and 28.6 mg/kg bw per day for female F_1 rats.] The feed containing gentian violet was certified to be within 10% of the target dose. For the interim evaluation at 24 months, there were 180 F_1 males and 180 F_1 females in the control group and 90 F_1 males and 90 F_1 females in each dose group. For the interim evaluation at 12 or 18 months, there were 15 F_1 males and 15 F_1 females in each group. Mortality was significantly increased in male rats at the intermediate dose, and there was a significant dose-related increase in mortality in female rats, with the increase in mortality being significant for females at the intermediate and highest dose. Survival was 121/180, 60/90, 47/90, and 55/90 in males, and 121/180, 56/90, 36/90 and 31/90 in females, for the control group and the groups at the lowest, intermediate, and highest dose, respectively. At 24 months, the terminal body weights of male and female rats receiving gentian violet at the highest dose were significantly lower than those of the controls [with the final mean body weights being 92% and 86% of those of the male and female control rats, respectively]. Complete necropsies and histopathological examinations were performed.

In male rats at 24 months, there was a significant positive trend in the incidence of hepatocellular adenoma ($P < 0.01$, Peto trend test), with incidence being significantly increased at the intermediate and highest dose (both $P < 0.01$, Peto test and Bonferroni correction). Such a significant positive trend was also observed for the incidence of follicular cell adenocarcinoma of the thyroid gland ($P < 0.01$, Peto trend test), with the incidence being significantly increased in rats at the lowest and the highest dose ($P < 0.05$ and $P < 0.01$, respectively, Peto test and Bonferroni correction). There was a significant positive trend in the incidence of follicular cell adenoma or adenocarcinoma (combined) of the thyroid gland [$P < 0.05$, Cochran–Armitage trend test], with incidence being significantly increased at the highest dose [$P < 0.01$, one-tailed Fisher exact test]. Mesothelioma of the testis or epididymis was observed with an incidence of 3%, 2%, 6%, and 9% in the control group and in the groups receiving the lowest, intermediate, and highest

dose, respectively [statistical analysis of the incidence of mesothelioma could not be performed, because the incidence was not reported as the number of rats with lesions per number of rats examined microscopically]. At 12 or 18 months, treatment did not cause a significant increase in the incidence of tumours in male rats. However, mesothelioma of the testis or epididymis was observed at 18 months with an incidence of 0%, 0%, 13%, and 13% in the control group and in groups at the lowest, intermediate, and highest dose, respectively [statistical analysis of the incidence of mesothelioma could not be performed because the incidence was not reported as the number of rats with lesions per number of rats examined microscopically].

In female rats, at 24 months, there was a significant positive trend in the incidence of follicular cell adenocarcinoma of the thyroid gland ($P < 0.01$, Peto trend test), and a significant increase in incidence at the two higher doses ($P < 0.05$ and $P < 0.01$, respectively, Peto test and Bonferroni correction). There was a significant positive trend in the incidence of follicular cell adenoma or adenocarcinoma (combined) of the thyroid gland [$P < 0.01$, Cochran–Armitage trend test], with a significant increase in incidence at the two higher doses [$P < 0.01$ and $P < 0.001$, respectively, one-tailed Fisher exact test]. Adenomas and adenocarcinomas of the clitoral gland were also observed with an incidence of 12%, 6%, 18%, and 33% in the control group and in groups at the lowest, intermediate, and highest dose, respectively [statistical analysis of the incidence of adenoma or adenocarcinoma (combined) of the clitoral gland could not be performed because the incidence was not reported as the number of rats with lesions per number of rats examined microscopically]. At 18 months, there was a significant positive trend in the incidence of mononuclear cell leukaemia ($P < 0.05$, Cochran–Armitage trend test), with a significant increase in incidence in females at the highest dose ($P < 0.01$, one-tailed Fisher exact test). At 12 months, no treatment-associated neoplasms were reported in females.

Regarding non-neoplastic lesions observed at 24 months, most were reported in the liver. Gentian violet caused a significant positive trend in the incidence and an increase in the incidence of hepatocyte regeneration and of mixed cell foci in all treated groups of male and female rats. Other lesions listed below also showed at least a significant positive trend in incidence, with incidence being significantly increased in one or two dose groups. In males, these other non-neoplastic lesions included clear cell foci, eosinophilic foci, basophilic foci, cytoplasmic vacuolization, and centrilobular necrosis of the liver, follicular cysts of the thyroid gland, red pulp hyperplasia of the spleen, and hyperplasia of the mesenteric lymph nodes. In females, these other non-neoplastic lesions included eosinophilic foci, haematopoietic cell proliferation, centrilobular fatty change and necrosis, and bile duct hyperplasia of the liver, and hyperplasia of the bone marrow. [The Working Group noted that this was a well-conducted study that complied with GLP, males and females were used, the duration of exposure and observation was adequate, and a high number of rats per group was used.]

3.2 Leucogentian violet

No studies were available to the Working Group.

3.3 Evidence synthesis for cancer in experimental animals

3.3.1 Gentian violet

The carcinogenicity of gentian violet has been assessed in male and female mice exposed by oral administration (in the feed) in one study, in male and female rats exposed in utero, followed by lactational exposure and oral administration

(in the feed) in another study, and in rats exposed by oral administration in a third study.

In one study that complied with GLP (NCTR, 1984; Littlefield et al., 1985), male and female B6C3F$_1$ mice were treated with gentian violet in the feed for up to 24 months. Gentian violet caused a significant increase, with a significant positive trend, in the incidence of hepatocellular adenoma and hepatocellular carcinoma in males and females at 24 months, and of hepatocellular adenoma in females at 18 months. In female mice, gentian violet caused significant increases, and significant positive trends, in the incidence of histiocytic sarcoma for the urinary bladder, ovaries, uterus, and vagina at 24 months. In males and females, there was a significant increase in the incidence of Harderian gland adenoma at 24 months.

In one study that complied with GLP (NCTR, 1988; Littlefield et al., 1989), male and female Fischer 344 rats were exposed to gentian violet in utero, followed by lactational exposure and oral administration (in the feed), for up to 24 months. In male and female rats, gentian violet caused a significant increase, and significant positive trend, in the incidence of follicular cell adenocarcinoma of the thyroid gland and follicular cell adenoma or adenocarcinoma (combined) of the thyroid gland at 24 months. In females, gentian violet caused a significant increase, and significant positive trend, in the incidence of mononuclear cell leukaemia at 18 months. In males, gentian violet caused a significant increase, and a significant positive trend, in the incidence of hepatocellular adenoma at 24 months.

A study in rats, where gentian violet was given by oral administration, was considered inadequate for the evaluation of the carcinogenicity of gentian violet in experimental animals (Kinosita, 1940).

3.3.2 Leucogentian violet

No studies were available to the Working Group.

4. Mechanistic Evidence

4.1 Absorption, distribution, metabolism, and excretion

4.1.1 Humans

No data were available to the Working Group.

4.1.2 Experimental systems

The absorption, distribution, metabolism, and excretion of gentian violet has been reviewed in Docampo & Moreno (1990), WHO (2014b), and OEHHA (2019).

(a) In vivo

Radiolabelled gentian violet was administered orally to rats and mice. Male and female Fischer 344 rats treated by gavage were given a single dose of [^{14}C]-labelled gentian violet (4.8 mg/kg bw for males, 5.2 mg/kg bw for females). The distribution of the [^{14}C]-labelled dye was measured in the liver, kidney, fatty tissue, gonads, muscle, urine, and faeces at 2, 4, 14, 24, and 36 hours after administration. Maximal residue levels were found at 4 hours in the liver, kidney, muscle, and gonads; a plateau was reached in fatty tissue after 24 hours. The depletion half-lives in male and female livers were 14.5 and 17.0 hours, respectively. The recovery values for males and females (males/females) were 2.2/2.2% and 72.9/63.8% of the single gentian violet dose in the urine and the faeces, respectively. In bile collected from cannulated rats, 5.7–6.4% of the single oral dose was recovered (McDonald et al., 1984a; NCTR, 1989).

Radiolabelled ([^{14}C]) gentian violet was also administered in multiple doses (twice per day for 7 days) to both male and female Fischer 344 rats

and B6C3F$_1$ mice by gavage. Maximal residue levels were found in fatty tissues of females of both species, and a statistically significant sex difference ($P < 0.01$) was noted. Residue levels in kidney and muscle tissues from both species, and in mouse livers, also showed sex differences. The recovery values for males and females (males/females) were 2.2%/1.6% and 65.5%/72.8% in the urine and the faeces of rats, respectively, and 5.9%/8.1% and 65.9%/67.4% in the urine and faeces of mice (McDonald et al., 1984a; NCTR, 1989).

Regarding the metabolism of gentian violet, McDonald & Cerniglia (1984) showed that leucogentian violet was excreted in the faeces collected from a female Fischer 344 rat that was given [^{14}C]-labelled gentian violet by gavage for 4 days. The metabolites of gentian violet were also analysed in mice and rats by NCTR (1989) and identified as three demethylated metabolites (pentamethyl *para*-rosaniline and *N*,*N*,*N*′,*N*′- and *N*,*N*,*N*′,*N*″-tetramethyl *para*-rosanilines) and two reduced metabolites (leucogentian violet and leuco-pentamethyl *para*-rosaniline). A summary of the proposed metabolism of gentian violet and leucogentian violet is provided in Fig. 4.1.

(b) In vitro

In bacteria, McDonald & Cerniglia (1984) demonstrated that gentian violet was transformed to leucogentian violet after incubation under anaerobic conditions with microflora isolated from human faeces, and from the intestinal contents of rats and chickens.

When metabolized by rat liver microsomes, gentian violet appears to undergo one-electron reduction by cytochrome P450 to produce a carbon-centred free radical (Harrelson & Mason, 1982). This carbon-centred radical can be formed by photoreduction of gentian violet after exposure to visible light (Docampo et al., 1988).

McDonald et al. (1984b) studied the metabolism of gentian violet in the presence of liver microsomes obtained from both sexes of four mouse strains, three rat strains, hamster, guinea-pig, and chicken: the main metabolites identified were pentamethyl *para*-rosaniline and the isomeric *N*,*N*,*N*′,*N*′- and *N*,*N*,*N*′,*N*″-tetramethyl *para*-rosanilines. Comparable patterns of demethylated metabolites were observed between species. [The Working Group noted that information about the relative amounts of the different metabolites, including leucogentian violet, was sparse.]

4.2 Evidence relevant to key characteristics of carcinogens

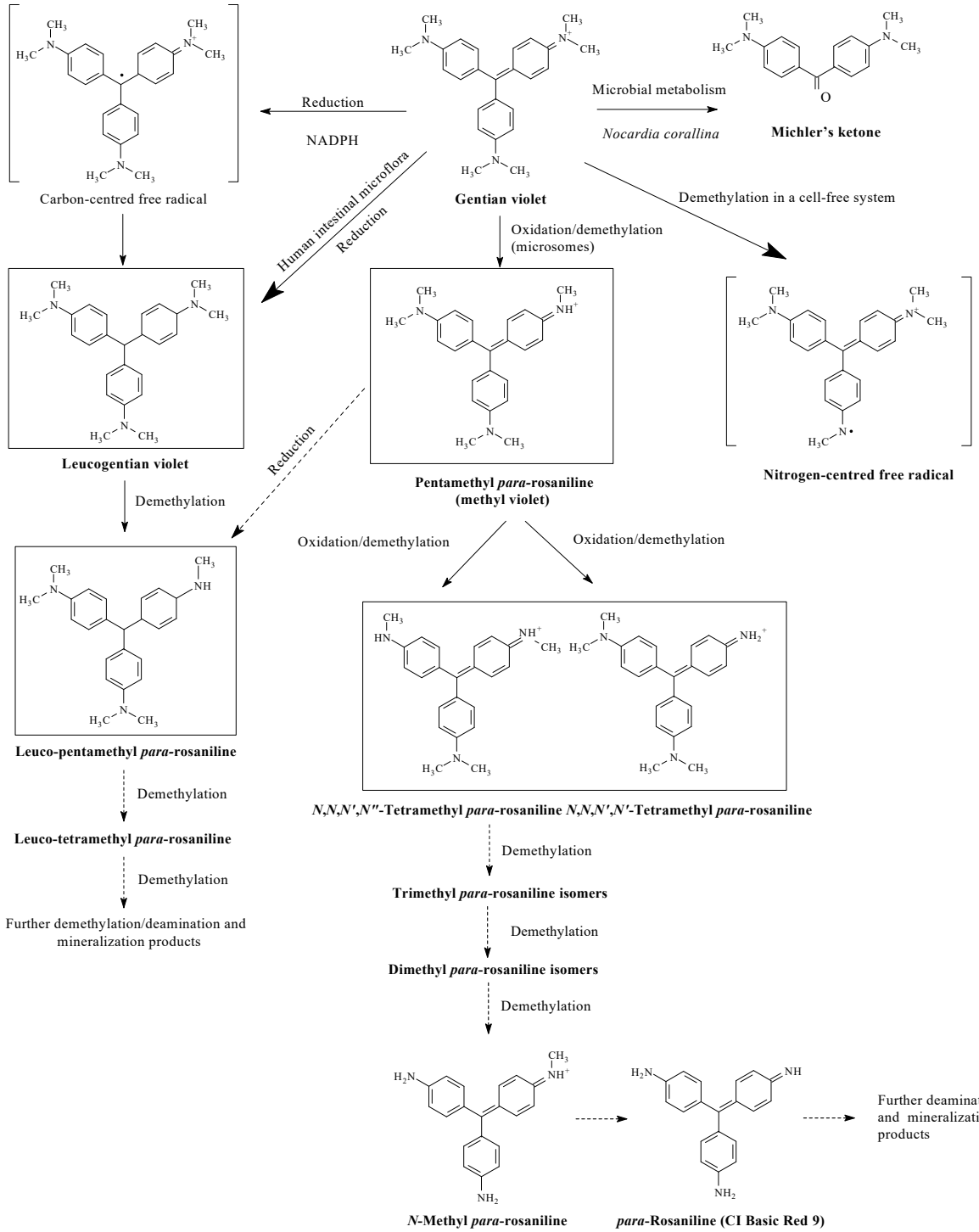
This section summarizes the evidence for the key characteristics of carcinogens (Smith et al., 2016), including whether gentian violet (and leucogentian violet) is electrophilic or can be metabolically activated to an electrophile; is genotoxic; or induces oxidative stress. Insufficient data were available for the evaluation of other key characteristics of carcinogens.

4.2.1 Is electrophilic or can be metabolically activated to an electrophile

Through measurement of sedimentation and viscosity, it was shown that gentian violet binds externally to the surface of the DNA helix, with a high degree of preference for two adjacent A–T base pairs, and that it induces severe bending accompanied by unwinding of the DNA helix (Müller & Gautier, 1975; Wakelin et al., 1981).

The ability of gentian violet to bind to bovine haemoglobin was demonstrated in vitro by Liu et al. (2013) using several spectroscopic and molecular modelling methods. A change in the spatial conformation of bovine haemoglobin was observed after binding of gentian violet (Liu et al., 2013).

Fig. 4.1 Metabolic pathways for gentian violet and leucogentian violet



Chemicals with structures in boxes were identified in the tissues of rats and mice treated with gentian violet. Reduced metabolites (leucogentian violet and leuco-pentamethyl *para*-rosaniline) were predominant. Arrows with solid lines indicate observed metabolic pathways; arrows with dashed lines indicate proposed pathways. Michler's ketone has been classified by IARC in Group 2B (*possibly carcinogenic to humans*).
NADPH, reduced form of nicotinamide adenine dinucleotide phosphate.
Created by the Working Group.

4.2.2 Is genotoxic

Table 4.1, Table 4.2, Table 4.3, and Table 4.4 summarize the available studies of the genetic and related effects of gentian violet.

(a) Humans

(i) Exposed humans

No data were available to the Working Group.

(ii) Human primary cells and human cell lines in vitro

See Table 4.1.

In human primary cells in vitro, a single concentration of gentian violet induced an increase in chromosomal aberration in cultured primary human peripheral blood lymphocytes from healthy donors (Au et al., 1978; Hsu et al., 1982), and from healthy individuals and patients with β-thalassaemia (Krishnaja & Sharma, 1995).

Au et al. (1978) also showed that gentian violet induced an increase in chromosomal aberrations in HeLa cells.

(b) Experimental systems

(i) Non-human mammals in vivo

See Table 4.2.

After injection of gentian violet in the tail veins of $B6C3F_1$ mice up to a dose of 8 mg/kg bw, no DNA damage was observed in splenic lymphocytes (Aidoo et al., 1990). Gentian violet also failed to induce chromosomal aberrations in bone marrow erythrocytes of Swiss albino mice that received the substance via drinking-water for 4 weeks up to a dose of 8 mg/kg (Au et al., 1979).

(ii) Non-human mammalian cells in vitro

See Table 4.3.

Aidoo et al. (1990) showed that gentian violet induced DNA damage (nucleoid sedimentation) in cultured lymphocytes from the spleens of $B6C3F_1$ mice and caused weak gene amplification in SV40-transformed Chinese hamster embryo (CO60) cells. Gentian violet induced DNA strand breaks in whole-blood samples collected from Sprague-Dawley rats (Díaz Gómez & Castro, 2013). When the rats were treated with antioxidants (α-tocopherol, lipoic acid, or *N*-acetylcysteine) before the blood samples were collected, the genotoxic effects induced by gentian violet were significantly decreased (Díaz Gómez & Castro, 2013). Gentian violet did not induce gene mutations at the hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) locus of Chinese hamster ovary (CHO) CHO-K1-BH4 cells, but caused a slight increase at the glutamic-pyruvate transaminase (*Gpt*) locus of CHO AS52 cells (the increase was observed only at very toxic concentrations, and was not reproduced with different gentian violet batches) (Aidoo et al., 1990).

Au et al. (1978) demonstrated that gentian violet induced mitotic anomalies. Gentian violet consistently induced chromosomal aberrations in various cell lines: *Mus musculus* mouse fibroblast L cells, a fibroblast cell line derived from *Peromyscus eremicus*, and a fibroblast cell line derived from the Indian muntjac (*Muntiacus muntjak*) (Au et al., 1978). It also induced chromosomal aberrations in CHO cells (Au et al., 1978, 1979; Au & Hsu, 1979). The cytogenic effect observed in CHO cells decreased in the presence of the S9 metabolic activation system (Au et al., 1979).

(iii) Non-mammalian experimental systems

See Table 4.4.

At low concentrations, gentian violet binds to double-stranded DNA at AT-rich regions, while it binds at all available sites at high concentrations (Fox et al., 1992).

Cornell K-strain chicken embryos treated with gentian violet did not show sister-chromatid exchange (Au et al., 1979; Bloom, 1984).

In one study performed on *Drosophila melanogaster*, gentian violet did not induce mutations

Table 4.1 Genetic and related effects of gentian violet in human primary cells and human cell lines in vitro

End-point	Tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Chromosomal aberration	HeLa cells (cervical cancer)	+	NT	5 µg/mL	5-h treatment; purity, NR	Au et al. (1978)
Chromosomal aberration	Blood peripheral lymphocytes	+	NT	5 µg/mL	5-h treatment; purity, NR	Au et al. (1978)
Chromosomal aberration	Blood peripheral lymphocytes	+	NT	20 µg/mL	Only one dose tested; purity, NR	Hsu et al. (1982)
Chromosomal aberration	Blood peripheral lymphocytes[b]	+	NT	1 µg/mL	Only one dose tested; purity, NR	Krishnaja & Sharma (1995)

h, hour; HIC, highest ineffective concentration; LEC, lowest effective concentration; NR, not reported; NT, not tested.

[a] +, positive.

[b] Lymphocytes collected from two groups (i.e. healthy individuals and patients with β-thalassaemia). Level of chromatid aberration in lymphocytes was similar in these two groups.

Table 4.2 Genetic and related effects of gentian violet in non-human mammals in vivo

End-point	Species, strain (sex)	Tissue, cell type	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
DNA damage (nucleoid sedimentation)	Mouse, $B6C3F_1$ (NR)	Spleen, lymphocytes	–	8 mg/kg	Injection in tail vein, 1 h before collection		Aidoo et al. (1990)
Chromosomal aberration	Mouse, Swiss albino (NR)	Bone marrow, erythrocytes	–	8 mg/kg	Drinking-water, 4 wk	GV dissolved at 20 and 40 µg/mL, and consumed dose calculated to be 4 and 8 mg/kg	Au et al. (1979)

GV, gentian violet; h, hour; HID, highest ineffective dose; LED, lowest effective dose; NR, not reported; wk, week.

[a] –, negative.

Table 4.3 Genetic and related effects of gentian violet in non-human mammalian cells in vitro

End-point	Species, tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
DNA damage (nucleoid sedimentation)	Mouse, B6C3F$_1$, spleen, lymphocytes	+	NT	1 µg/mL	1-h treatment	Aidoo et al. (1990)
DNA strand breaks (comet assay)	Rat, blood, leukocytes	+	NT	250 µg/mL[b]	24 and 48 h	Díaz Gómez & Castro (2013)
Gene amplification	Chinese hamster, embryo, C060 (SV40-transformed)	(+)	NT	0.125 µg/mL	5-h treatment; weak DNA amplification observed	Aidoo et al. (1990)
Gene mutation (*Hprt* locus)	Chinese hamster, ovary, CHO-K1-BH4	–	–	1 µg/mL	5-h treatment	Aidoo et al. (1990)
Gene mutation (*Gpt* locus)	Chinese hamster, ovary, CHO-AS52	(+)	–	1.5 µg/mL	5-h treatment; increase observed only at very toxic concentrations, and not always reproduced	Aidoo et al. (1990)
Mitotic anomalies[c]	Chinese hamster, ovary, CHO	+	NT	10 µg/mL	2-h treatment; purity, NR	Au et al. (1978)
Chromosomal aberration	Mouse, fibroblast L cells	+	NT	5 µg/mL	5-h treatment; purity, NR	Au et al. (1978)
Chromosomal aberration	*Peromyscus eremicus*, NR, fibroblasts	+	NT	5 µg/mL	5-h treatment; purity, NR	Au et al. (1978)
Chromosomal aberration	Indian muntjac, NR, fibroblasts	+	NT	5 µg/mL	5-h treatment; purity, NR	Au et al. (1978)
Chromosomal aberration	Chinese hamster, ovary, CHO	+	NT	5 µg/mL	5-h treatment; purity, NR	Au et al. (1978)
Chromosomal aberration	Chinese hamster, ovary, CHO	+	+	5 µg/mL (–S9); 10 µg/mL (+S9)	5-h treatment; S9 decreased the clastogenic effect; purity, NR	Au et al. (1979)
Chromosomal aberration	Chinese hamster, ovary, CHO	+	NT	10 µM [4 µg/mL]	Only one dose tested; purity, NR	Au & Hsu (1979)

CHO, Chinese hamster ovary; *Gpt*, glutamic-pyruvate transaminase; h, hour; HIC, highest ineffective concentration; *Hprt*, hypoxanthine-guanine phosphoribosyltransferase; LEC, lowest effective concentration; NR, not reported; NT, not tested; S9, 9000 × *g* supernatant.

[a] +, positive; –, negative; (+), positive in a study of limited quality.

[b] Calculated from the data provided in the article (50 µL of gentian violet solution at 0.0245 M for 2 mL of blood; the relative molecular mass of gentian violet is 408).

[c] Mitotic anomalies include increase in mitotic index, metaphase : anaphase ratio and frequency of anaphase abnormalities (chromatin bridges, lagging chromosomes, chromosome fragments, and sticky chromosomes).

Table 4.4 Genetic and related effects of gentian violet in non-mammalian experimental systems

Test system (species, strain)	End-point	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Cornell K-strain chicken (NR), embryo	Sister-chromatid exchange	–	NA	100 µg/embryo	Test solution applied to inner shell membrane (after removing the portion of the shell overlying the air cell) Purity, NR	Au et al. (1979)
Cornell K-strain chicken (NR), embryo	Sister-chromatid exchange	–	NA	"Amounts in the range of 10–100 µL are used"	Air-cell method (test solution is dropped onto the inner shell membrane after removing the portion of the shell overlying the air cell) [No more details given on the amount of GV used (10–100 µL)] Purity, NR	Bloom (1984)
Drosophila melanogaster	Sex-linked recessive lethal assay	–	NA	500 ppm (feed) or 550 ppm (injected)	Purity, 92%	Mason et al. (1992)
Bacillus subtilis (rec assay)	DNA damage, differential toxicity	+	NT	2 mg/0.02 mL	Only one dose tested Purity, NR	Fujita et al. (1976)
Bacillus subtilis BD224 (rec assay)	DNA damage, differential toxicity	NT	+	200 µg/plate	Purity, 80–95%	Choudhary et al. (2004)
Escherichia coli B	DNA strand breaks	+	NT	~10 µM [4 µg/mL]	Purity, NR	Grigg et al. (1984)
Salmonella typhimurium TA1535, TA100 (base substitution, at GC)	Reverse mutation	–	NT	4 µg/plate	Purity, NR	Shahin & Von Borstel (1978)
Salmonella typhimurium TA98, TA1538 (frameshift +1)	Reverse mutation	–	NT	4 µg/plate	Purity, NR	Shahin & Von Borstel (1978)
Salmonella typhimurium TA1537 (frameshift –1)	Reverse mutation	–	NT	4 µg/plate	Purity, NR	Shahin & Von Borstel (1978)
Salmonella typhimurium TA1535, TA100 (base substitution, at GC)	Reverse mutation	–	–	50 µg/plate	Purity, NR	Au et al. (1979)
Salmonella typhimurium TA98 (frameshift +1)	Reverse mutation	–	–	50 µg/plate	Purity, NR	Au et al. (1979)
Salmonella typhimurium TA1537 (frameshift –1)	Reverse mutation	–	–	50 µg/plate	Purity, NR	Au et al. (1979)

Table 4.4 (continued)

Test system (species, strain)	End-point	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium TA98 (frameshift +1)	Reverse mutation	+	+	16 µg/plate	Data provided for only one dose; purity, NR	Fujita et al. (1976)
Salmonella typhimurium TA100 (base substitution, at GC)	Reverse mutation	-	+	16 µg/plate	Data provided for only one dose; purity, NR	Fujita et al. (1976)
Salmonella typhimurium TA1535 (base substitution, at GC)	Reverse mutation	(+)	–	0.32 µg/plate	Reproducible increase observed only in TA1535 at middle dose of 0.32 µg/plate; purity, 97%	Bonin et al. (1981)
Salmonella typhimurium TA100 (base substitution, at GC)	Reverse mutation	–	–	3.2 µg/plate	Purity, 97%	Bonin et al. (1981)
Salmonella typhimurium TA98, TA1538 (frameshift +1)	Reverse mutation	–	–	3.2 µg/plate	Purity, 97%	Bonin et al. (1981)
Salmonella typhimurium TA1537 (frameshift –1)	Reverse mutation	–	–	3.2 µg/plate	Purity, 97%	Bonin et al. (1981)
Salmonella typhimurium TA100 (base substitution, at GC)	Reverse mutation	–	–	50 µg/plate	Purity, NR	Levin et al. (1982)
Salmonella typhimurium TA98 (frameshift +1)	Reverse mutation	–	–	50 µg/plate	Purity, NR	Levin et al. (1982)
Salmonella typhimurium TA1537 (frameshift –1)	Reverse mutation	–		50 µg/plate	Purity, NR	Levin et al. (1982)
Salmonella typhimurium TA1535 (base substitution, at GC)	Reverse mutation	–	–	0.5 µg/plate	Purity, NR	Thomas & MacPhee (1984)
Salmonella typhimurium TA100 (base substitution, at GC)	Reverse mutation	–	–	50 µg/plate	Purity, NR	Hass et al. (1986)
Salmonella typhimurium TA98 (frameshift +1)	Reverse mutation	–	–[b]	50 µg/plate	Purity, NR	Hass et al. (1986)
Salmonella typhimurium TA97 (frameshift –1)	Reverse mutation	–	–	50 µg/plate	Purity, NR	Hass et al. (1986)

Table 4.4 (continued)

Test system (species, strain)	End-point	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium TA100 (base substitution, at GC)	Reverse mutation	–	–	10 µg/plate	Purity, > 97% or > 99%	Aidoo et al. (1990)
Salmonella typhimurium TA98 (frameshift +1)	Reverse mutation	–	–	10 µg/plate	Purity, > 97% or > 99%	Aidoo et al. (1990)
Salmonella typhimurium TA97 (frameshift –1)	Reverse mutation	–	(+)[c]	0.5 µg/plate	Increase slightly > 2-fold for GV purity, 97%; increase < 2-fold for purity, 99%	Aidoo et al. (1990)
Salmonella typhimurium TA104 (base substitution, at AT)	Reverse mutation	–	(+)[c]	0.5 µg/plate	Increase slightly > 2-fold for GV purity, 99%; increase < 2-fold for purity, 97%	Aidoo et al. (1990)
Salmonella typhimurium TA100 (base substitution, at GC)	Reverse mutation	–	–	25 µg/plate	Purity, NR	Malachová et al. (2006)
Salmonella typhimurium TA98 (frameshift +1)	Reverse mutation	–	(+)	25 µg/plate	Purity, NR; GV was highly toxic at 25 µg/plate	Malachová et al. (2006)
Salmonella typhimurium YG1041 (frameshift)	Reverse mutation	–	–	25 µg/plate	Purity, NR	Malachová et al. (2006)
Salmonella typhimurium YG1042 (base substitution)	Reverse mutation	–	–	25 µg/plate	Purity, NR	Malachová et al. (2006)
Salmonella typhimurium TA100-lux (base substitution, at GC)	Reverse mutation	–	–	100 µg/plate	Purity, NR	Ackerman et al. (2009)
Salmonella typhimurium TA98-lux (frameshift +1)	Reverse mutation	–	–	100 µg/plate	Purity, NR	Ackerman et al. (2009)
Salmonella typhimurium TA98 (frameshift +1)	Reverse mutation	NT	(+)	100 µg/plate	Analytical grade; fold-increase slightly lower than 2; only one dose tested	Ayed et al. (2017)
Escherichia coli DG1669 (frameshift)	Reverse mutation	+	+	25 µg/plate	Purity, NR	Thomas & MacPhee (1984)
Escherichia coli WP2s (base substitution, at AT)	Reverse mutation	+	+	5 µmol/L [2 µg/mL]	Purity, NR	Hass et al. (1986)
Escherichia coli WP2	Reverse mutation	+	NT	80 µg/plate	Purity, NR	Fujita et al. (1976)
Escherichia coli W3110 polA$^+$, mutant p3478 polA$^-$	Rosenkranz repairable DNA assay	+	+	10 µg/plate	Purity, NR	Au et al. (1979)

Table 4.4 (continued)

Test system (species, strain)	End-point	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Escherichia coli W3110 polA$^+$, mutant p3478 polA$^-$	Rosenkranz repairable DNA assay	+	+	10 µg/plate	Purity, NR	Levin et al. (1982)
Saccharomyces cerevisiae XV185-14C	Rosenkranz repairable DNA assay	–	NT	8 µg/plate	Purity, NR	Shahin & Von Borstel (1978)

GV, gentian violet; HIC, highest ineffective concentration; LEC, lowest effective concentration; NA, not applicable; NR, not reported; NT, not tested; ppm, parts per million.

[a] +, positive; –, negative; (+), positive in a study of limited quality.

[b] Hass et al. (1986) also reported that the metabolite of GV, leucogentian violet, was positive in *Salmonella typhimurium* TA98 at 50 µg/plate in the presence of metabolic activation. Purity was not reported.

[c] Aidoo et al. (1990) also showed that the major metabolites of GV, pentamethyl-*para*-rosaniline, *N*,*N*,*N*′,*N*′-tetramethyl-*para*-rosaniline, and *N*,*N*,*N*′,*N*″-tetramethyl-*para*-rosaniline, induced a dose-related increase in the number of mutant colonies in *Salmonella typhimurium* TA97, which reached 1.5-, 1.7-, and 1.4-fold, respectively, compared with the control.

in a sex-linked recessive lethal assay (Mason et al., 1992).

Gentian violet induced DNA damage in *Bacillus subtilis* (Fujita et al., 1976; Choudhary et al., 2004). Grigg et al. (1984) observed that gentian violet induced DNA strand breaks in *Escherichia coli* B strain.

An overwhelming majority of the data show that gentian violet did not induce mutagenicity with or without metabolic activation in *Salmonella typhimurium* strains TA1535, TA100, TA1538, TA97, TA98, TA1537, YG1041, and YG1042 (Shahin & Von Borstel, 1978; Au et al., 1979; Bonin et al., 1981; Levin et al., 1982; Thomas & MacPhee, 1984; Hass et al., 1986; Aidoo et al., 1990; Malachová et al., 2006; Ackerman et al., 2009). However, a few authors reported a mutagenic effect without metabolic activation in TA98, TA100, and TA1535 (Fujita et al., 1976; Bonin et al., 1981), with metabolic activation in TA98 and TA100 (Fujita et al., 1976; Ayed et al., 2017), as well as in TA97 and TA104 (Aidoo et al., 1990). Malachová et al. (2006) described a mutagenic effect of crystal [gentian] violet with metabolic activation in TA98, which was associated with a cytotoxic effect. In *E. coli* strains, gentian violet caused mutagenicity with and without metabolic activation (Thomas & MacPhee, 1984; Hass et al., 1986), and induced mutagenicity without metabolic activation in a study by Fujita et al. (1976) (not tested with metabolic activation). In the Rosenkranz repairable DNA assay, gentian violet gave positive results in *E. coli* strains W3110 polA$^+$ and P3478 polA$^-$ (Au et al., 1979; Levin et al., 1982), but negative results in the *Saccharomyces cerevisiae* XV185-14C strain (Shahin & Von Borstel, 1978).

4.2.3 Induces oxidative stress

As already mentioned above (Section 4.1.2.b), gentian violet can lead to the formation of a carbon-centred free radical, either by photoreduction (Reszka et al., 1986; Docampo et al., 1988) or by enzymatic reaction (Harrelson & Mason, 1982).

4.3 Other relevant evidence

4.3.1 Humans

Several studies using patch tests showed that gentian violet was among the least active sensitizers of several tested drugs, because contact hypersensitivity was rarely observed with gentian violet (Bajaj et al., 1982; Pasricha & Gupta, 1982; Bajaj & Gupta, 1986; Mahaur et al., 1987).

Bielicky & Novák (1969) observed that, in patients with eczema, gentian violet induced sensitization. Moreover, cross-sensitization between crystal violet and malachite green was possible, as the probable determinant groups for sensitization are -N$(CH_3)_2$ and -N$(C_2H_5)_2$.

4.3.2 Experimental systems

No data were available to the Working Group.

4.4 Data relevant to comparisons across agents and end-points

The mechanistic characteristics common to carcinogens (the 10 key characteristics of carcinogens) can be investigated through biochemical and cell-based assays run by the United States Environmental Protection Agency (US EPA) and the United States National Institutes of Health Toxicity Forecaster/Toxicology in the 21st Century (ToxCast/Tox21) high-throughput screening programmes (Chiu et al., 2018; Guyton et al., 2018). Since 2017, the *IARC Monographs* have described the results of high-throughput screening assays to compare activity across agents and other in vitro and in vivo evidence relevant to the key characteristics.

Of the five compounds included in *IARC Monographs* Volume 129, three have been evaluated in at least some of the US EPA and

United States National Institutes of Health high-throughput screening assays: gentian violet (CAS No. 548-62-9), malachite green (malachite green chloride, CAS No. 569-64-2, and malachite green oxalate, CAS No. 2437-29-8), and leucomalachite green (CAS No. 129-73-7) (US EPA, 2020a, b, c, d). Table 4.5 summarizes findings for assay end-points mapped to key characteristics for the compounds evaluated. Details of the specific assays (and end-points) run for each chemical in this volume and the mapping to the key characteristics can be found in the Supplementary Material (Annex 1, Supplementary material for Section 4, web only; available from: https://www.publications.iarc.fr/603). It is important to note that some assays either lacked, or had uncharacterized and generally low, xenobiotic metabolism, limiting observations primarily to effects elicited by parent compounds. The strengths of the high-throughput screening battery of assays are the standardization of the protocols applied across compounds, allowing comparisons across compounds and the evaluation of specificity of assay end-points to the key characteristics, and ultimately to the apical outcome of carcinogenesis (Becker et al., 2017; Chiu et al., 2018; Watford et al., 2019). The 299 ToxCast/Tox21 assay end-points mapped to key characteristics interrogated in this and other monographs are initially described in Chiu et al. (2018), with the most up-to-date mapping described in detail in *IARC Monographs* Volume 123 (IARC, 2019). All ToxCast/Tox21 data were downloaded from the US EPA CompTox Chemicals Dashboard 10th Release (US EPA, 2021) between 2 and 19 October 2020 or on 24 February 2021 (malachite green oxalate).

The individual assessments for each compound are included in the corresponding monographs in the present volume.

4.4.1 Gentian violet

Results were available for 280 assay end-points (out of the 299 that were mapped to key characteristics) for gentian violet (US EPA, 2020a). Gentian violet was considered active in 126 assay end-points, including the one assay end-point mapped to "is electrophilic or can be metabolically activated to an electrophile", 10 of the 12 assay end-points mapped to "is genotoxic", 2 of the 5 mapped to "induces epigenetic alterations", 8 of 16 end-points mapped to "induces oxidative stress", 27 of the 90 assay end-points mapped to "modulates receptor-mediated effects", and 78 of 109 end-points mapped to "alters cell proliferation, cell death, or nutrient supply".

Assays within the "is genotoxic" key characteristic provide measurements of DNA damage or repair in human liver (HepG2), kidney (HEK293T), and intestinal (HCT-116) cell lines, as well as a CHO cell line (CHO-K1) and a chicken lymphoblast cell line (DT40). Gentian violet (purity, > 90%) elicited TP53 activation measured through reporter assays in HCT-116 and HepG2 cells. Gentian violet was considered active, as measured by phosphorylated histone H2AX (γH2AX) assay detecting H2AX protein phosphorylation, consistent with DNA double-strand breaks in a CHO cell line (CHO-K1). Gentian violet was also considered active as measured by assays using DT40 chicken lymphoblastoid cell lines deficient for the DNA-repair genes *REV3* and *KU70/RAD54*. Gentian violet was not considered active as determined by the ATAD5-luc assay in HEK293T cells, which measures levels of ATAD5 protein that localize to the site of stalled replication forks resulting from DNA damage in replicating cells. It is important to note that both positive (e.g. etoposide, 5-fluorouridine, tetra-*N*-octylammonium bromide, and mitomycin C) and negative (dimethyl sulfoxide) controls are run concurrently, and subsequent analyses and activity calls are

Table 4.5 Summary of results of ToxCast/Tox21 high-throughput screening assays linked to key characteristics of carcinogens for agents reviewed in *IARC Monographs* Volume 129[a]

Key characteristic (total number of assays mapped to characteristic)[b]	No. of positive results out of the number of assays			
	Gentian violet (CAS No. 548-62-9)	Malachite green chloride (CAS No. 569-64-2)	Malachite green oxalate (CAS No. 2437-29-8)	Leucomalachite green (CAS No. 129-73-7)
1. Is electrophilic or can be metabolically activated (2)	1 out of 1[c]	NT	1 out of 1	0 out of 1
2. Is genotoxic (12)	10 out of 12	1 out of 2	8 out of 9	2 out of 10
4. Induces epigenetic alterations (5)	2 out of 5	5 out of 5	1 out of 1	0 out of 5
5. Induces oxidative stress (16)	8 out of 16	4 out of 10	3 out of 4	4 out of 13
6. Induces chronic inflammation (47)	0 out of 47	0 out of 46	0 out of 1	1 out of 47
8. Modulates receptor-mediated effects (98)	27 out of 90	17 out of 50	22 out of 32	13 out of 69
10. Alters cell proliferation, death, or nutrient supply (119)	78 out of 109	40 out of 63	56 out of 58	24 out of 91
Total hits out of total no. of assays evaluated	126 out of 280	67 out of 176	91 out of 106	44 out of 236

CAS, Chemical Abstracts Service; NT, not tested; Tox21, Toxicology in the 21st Century programme; ToxCast, Toxicity Forecaster programme.

[a] No high-throughput screening data were available for leucogentian violet (CAS No. 603-48-5) and CI Direct Blue 218 (CAS No. 28407-37-6).

[b] Seven of the 10 key characteristics have mapped high-throughput screening assay end-points, as described by Chiu et al. (2018). The mapping file with findings for *IARC Monographs* Volume 129 chemicals is available in the Supplementary Material (Annex 1, Supplementary material for Section 4, web only; available from: https://www.publications.iarc.fr/603). No assay end-points in ToxCast or Tox21 were determined to be applicable to the evaluation of three key characteristics including causes immortalization, alters DNA repair or causes genomic instability, and is immunosuppressive.

[c] Indicates the number of positive results out of the number of assays tested for that chemical.

normalized against data for positive and negative controls run on the same plates (Hsieh et al., 2019).

4.4.2 Leucogentian violet

Leucogentian violet has not been evaluated in high-throughput screening assays.

4.4.3 Summary

Gentian violet has been evaluated in ToxCast or Tox21 assays with end-points mapped to key characteristics of carcinogens. It was active in a significant fraction of mapped end-points in which it had been tested (45%). Specifically, gentian violet was considered active in most of the "is genotoxic" assay end-points. It was also considered active for a variety of the assay end-points mapped to the following key characteristics: induces epigenetic alterations, induces oxidative stress, modulates receptor-mediated effects, and alters cell proliferation, cell death, or nutrient supply. Relevant to findings in other sections, gentian violet was considered active in an assay measuring thyroid receptor antagonism in GH3, a rat pituitary gland cell line, and was considered to give negative results in an assay measuring thyroid hormone receptor-agonist activity in the same cell line. Gentian violet was considered to give negative results in an assay measuring thyroid hormone receptor-mediated transcription in HepG2 cells. Leucogentian violet has not been evaluated in high-throughput screening assays.

5. Summary of Data Reported

5.1 Exposure characterization

Gentian violet is a cationic triphenylmethane dye. The reduced form of gentian violet is leucogentian violet, which can be formed by chemical or enzymatic reduction of gentian violet. Gentian violet is widely used as a textile dye, a pigment for consumer and industrial products (inks, papers, and coatings), as a biological stain (Gram stain), and for cosmetic purposes (hair dyes and body piercing). The antibacterial, antifungal, and anthelmintic properties of gentian violet make it an important agent in human medicine as an antiseptic to prevent infection and promote wound healing, and as a topical treatment for fungal and bacterial infections. Gentian violet also has several veterinary applications for the treatment of fungal and parasite disease in fish, disinfection of aquariums, topical treatment for bacterial and fungal infections in livestock, and the prevention of growth of mould and fungi in poultry feeds. Leucogentian violet is a precursor in the production of gentian violet dye, and is used as an analytical reagent to enhance blood impression evidence in forensic analysis, for laboratory determination of anions and metal ions, and as a radiochromic indicator in dosimeters to detect radiation exposure. As gentian violet may be used to control fish diseases, residues of its major metabolite, leucogentian violet, might be found in treated fish or shellfish, and have a longer residence time than the parent compound.

Gentian violet may be released into the environment from waste discharged by textile mills and by other industrial processing, and persists in soil and aquatic species primarily as leucogentian violet.

Overall, data on exposure to gentian violet and leucogentian violet are sparse. The potential for occupational exposure to gentian violet and leucogentian violet exists through dermal contact and inhalation at workplaces where the compound is produced or applied; however, no current data on exposed occupational populations or occupational exposure levels were identified.

In the general population, exposure can occur through contact with textiles, paper, and inks

containing gentian violet, medicinal or ornamental fish treatment, cosmetic application for hair dyeing and body piercing, and through the consumption of drinking-water, fish, or shellfish containing residues of gentian violet and leucogentian violet. One study indicated that drinking-water may be an important route of exposure to gentian violet.

Gentian violet is listed by the European Chemicals Agency as a carcinogen (Category 2) and is a substance of very high concern. Gentian violet is not authorized for use as a veterinary drug or for cosmetic applications in many countries, and there is zero tolerance for residues of gentian violet, or its marker leucogentian violet, in food for human consumption.

5.2 Cancer in humans

No data were available to the Working Group.

5.3 Cancer in experimental animals

5.3.1 Gentian violet

Exposure to gentian violet caused an increase in the incidence of malignant neoplasms in both sexes of two species (mouse and rat).

In $B6C3F_1$ mice exposed to gentian violet in the feed, there was a significant positive trend and significant increase in the incidence of hepatocellular carcinoma in males and females, and of histiocytic sarcoma of the urinary bladder, ovaries, uterus, and vagina in females in a study that complied with Good Laboratory Practice (GLP).

In Fischer 344 rats exposed to gentian violet in utero, followed by lactational exposure and oral administration (in the feed), there was a significant positive trend and significant increase in the incidence of follicular cell adenocarcinoma of the thyroid gland in males and females, and of mononuclear cell leukaemia in females in a study that complied with GLP.

5.3.2 Leucogentian violet

No studies were available to the Working Group.

5.4 Mechanistic evidence

No data on absorption, distribution, metabolism, or excretion in humans were available. In mice and rats, orally administered gentian violet is distributed to the liver, kidney, and fatty tissue, and is excreted primarily in faeces. Various demethylated and reduced metabolites have been detected in rats and mice, and in vitro experiments using microsomal preparations from different species. Bacteria have been shown to transform gentian violet to the metabolite leucogentian violet, but data from mammalian species are sparse.

For gentian violet, the mechanistic evidence is suggestive but incoherent across studies in experimental systems, and no data in humans were available. Regarding the key characteristics of carcinogens, gentian violet binds to isolated DNA and to haemoglobin, but no data on DNA adducts were available. Gentian violet induced chromosomal aberrations in human primary cells and in various cultured mammalian cell lines in a few studies. It was considered active in various high-throughput in vitro assays indicative of DNA damage including TP53 activation and γH2AX. However, it did not induce DNA damage or chromosomal aberrations in orally exposed mice in the few studies available. In rodent cells in vitro, it induced DNA damage but not gene mutations. It gave negative results in tests in chicken embryos and in *Drosophila melanogaster*, and largely negative results across various *Salmonella typhimurium* strains, including TA1535, TA100, TA1538, TA97, TA98, TA1537, TA104, YG1041, and YG1042. In *Escherichia coli* strains, gentian violet caused mutagenicity with and without metabolic activation. For other key

characteristics of carcinogens, there is a paucity of available data.

For leucogentian violet, data were scarce.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *inadequate evidence* in humans regarding the carcinogenicity of gentian violet.

There is *inadequate evidence* in humans regarding the carcinogenicity of leucogentian violet.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of gentian violet.

There is *inadequate evidence* in experimental animals regarding the carcinogenicity of leucogentian violet.

6.3 Mechanistic evidence

For gentian violet, there is *limited* mechanistic evidence.

For leucogentian violet, there is *inadequate* mechanistic evidence.

6.4 Overall evaluation

Gentian violet is *possibly carcinogenic to humans (Group 2B).*

Leucogentian violet is *not classifiable as to its carcinogenicity to humans (Group 3).*

6.5 Rationale

The *Group 2B* evaluation for gentian violet is based on *sufficient evidence* for cancer in experimental animals. The evidence regarding cancer in humans is *inadequate* as no studies were available. The mechanistic evidence is *limited* for gentian violet, based on suggestive but incoherent evidence in experimental systems pertinent to key characteristics of carcinogens. The *sufficient evidence* for cancer in experimental animals is based on an increase in the incidence of malignant neoplasms in males and females of two species in two studies that comply with GLP.

Leucogentian violet was evaluated as *Group 3* because the evidence regarding cancer in humans and in experimental animals, as well as mechanistic evidence, is *inadequate*, since no studies were available.

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MALACHITE GREEN AND LEUCOMALACHITE GREEN

1. Exposure Characterization

1.1 Identification of the agent

Malachite green is a cationic triphenylmethane dye that occurs as a chloride but is also available as an oxalate and as other salts. The name "malachite green" refers to the coloured cation and is used interchangeably for the chloride, oxalate, and other salts in the exposure characterization literature, often with no identification of the salt being made. The malachite green cation has a pH-dependent equilibrium with the corresponding carbinol form. The reduced form of malachite green is leucomalachite green, which can be formed by chemical or enzymatic reduction of malachite green chloride, malachite green oxalate, and other malachite green salts. Malachite green and its leuco base are susceptible to oxidation–reduction and demethylation reactions.

1.1.1 Malachite green

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 569-64-2/14426-28-9 (chloride); 2437-29-8 (oxalate); 10309-95-2 (cation); 510-13-4 (carbinol base); 41272-40-6 (acetate); 16044-24-9 (hydrogen sulfate); 68527-61-7 (benzoate) (NLM, 2020)

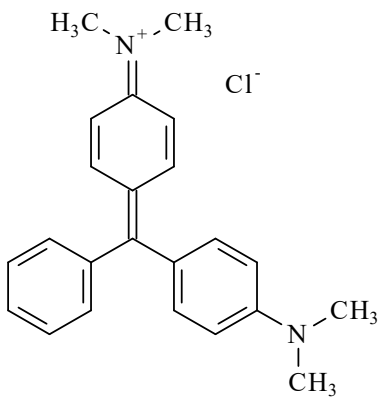
Chem. Abstr. Serv. name: malachite green (American Chemical Society, 2021a); methanaminium,*N*-[4-[[4-(dimethylamino)phenyl]phenylmethylene]-2,5-cyclohexadien-1-ylidene]-*N*-methyl-chloride (1:1) (ECHA, 2020a)

EC No.: 209-322-8

IUPAC systematic name: [4-[[4-(dimethylamino)phenyl]-phenylmethylidene]cyclohexa-2,5-dien-1-ylidene]-dimethylazanium chloride (NCBI, 2020a)

Synonyms: Basic Green 4, China Green, Victoria Green B, Aniline Green, Diamond Green B, Benzal Green, Benzaldehyde green, CI 42000, Magentagreencrystals, Aizen Malachite Green (NCBI, 2020a).

(b) Structural and molecular formulae, and relative molecular mass



Molecular formula: $C_{23}H_{25}ClN_2$

Relative molecular mass: 364.91

(c) Chemical and physical properties of the pure substance

Description: green crystals with metallic lustre; water solutions are blue-green (NCBI, 2020b)

Boiling point: 452 °C (predicted) (US EPA, 2020a)

Melting point: 180 °C (predicted) (US EPA, 2020a)

Density: 1.03 g/cm³ at 20 °C (predicted) (US EPA, 2020a)

Solubility: 4.00 × 104 mg/L at 25 °C in water; very soluble in ethanol; soluble in methanol and amyl alcohol (NCBI, 2020b)

Dissociation constant (of the conjugated acid BH^+): $pK_a = 6.9$ (at 25 °C) (Goldacre & Philips, 1949); in aqueous solutions, malachite green occurs in an equilibrium between a green ionic form (i.e. dye salt) and a colourless hydrated derivative (malachite green carbinol or pseudobase). The rate of carbinol formation is a function of the pH. In strongly acidic solutions (pH < 1), the colour changes to yellow as malachite green is converted to a dication. In alkaline solutions (pH > 12), the green colour is lost due to hydration of the central carbon atom and formation of the carbinol. The increase in temperature increases the rate of carbinol formation (El Hajj Hassan et al., 2011; Cooksey, 2016). The carbinol is relatively insoluble in water (~500 pg/L) and it is more lipophilic than the cationic form (Culp & Beland, 1996).

Vapour pressure: 2.4×10^{-13} mm Hg at 25 °C (estimated) (NCBI, 2020b); 3.22×10^{-7} mm Hg at 25 °C (predicted) (US EPA, 2020a)

Flash point: 238 °C (predicted) (US EPA, 2020a)

Stability and reactivity: neutralizes acids in exothermic reactions to form salts plus water; incompatible with isocyanates, halogenated organics, peroxides, phenols (acidic), epoxides, anhydrides, and acid halides; in combination with strong reducing agents, such as hydrides, flammable gaseous hydrogen may be generated (NCBI, 2020a)

Octanol/water partition coefficient (P): log $K_{ow} = 0.62$ (NCBI, 2020b)

Henry's law constant: 1.93×10^{-14} atm m³ mol^{-1} [3.10×10^{-11} Pa m³ mol^{-1}] (estimated) at 25 °C (NLM, 2021)

Ultraviolet maximum: 617 nm (NCBI, 2020b).

(d) Impurities

The purity of malachite green may range from 70% to 98% (ECHA, 2010). The main impurities of malachite green are monodesmethyl malachite green (1.62–3.8%), leucomalachite green (1–7.5%), monodesmethyl leucomalachite green (0.5%), malachite green carbinol (0.19%), 4-(dimethylamino)benzophenone (0.76%), and methanol (1.4%) (Culp et al., 1999, 2006; Le Goff & Wood, 2008).

1.1.2 Leucomalachite green

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 129-73-7

Chem. Abstr. Serv. name: leucomalachite green (American Chemical Society, 2021b); benzenamine, 4,4′-(phenylmethylene)bis[*N*,*N*-dimethylbenzeneamine] (ECHA, 2020b)

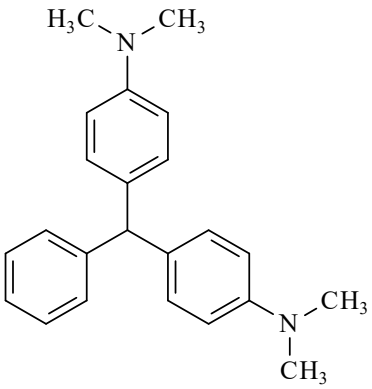
EC No.: 204-961-9

IUPAC systematic name: 4-[[4-(dimethylamino)phenyl]-phenylmethyl]-*N*,*N*-dimethylaniline (NCBI, 2020c)

Synonyms: malachite green leuco, malachite green leuco base, Leuco malachite green, 4,4′-bis(dimethylamino)triphenyl-

methane, tetramethyldiaminotriphenylmethane, CI Basic Green 4, leuco base, *N*,*N*,*N*′,*N*′-tetramethyl-4,4′-benzylidenedianiline (NCBI, 2020c)

(b) Structural and molecular formulae, and relative molecular mass



Molecular formula: $C_{23}H_{26}N_2$

Relative molecular mass: 330.47

(c) Chemical and physical properties of the pure substance

Description: off-white to light-brown powder (NCBI, 2020d)

Boiling point: 414 °C (predicted) (US EPA, 2020b)

Melting point: 101 °C (US EPA, 2020b)

Density: 1.06 g/cm³ (at 20 °C) (predicted) (US EPA, 2020b)

Solubility: 6.40×10^{-2} mg/L at 25 °C in water (estimated); very soluble in benzene and ethyl ether; 30 mg/mL in ethyleneglycol monomethyl ether; 4 at 25 °C mg/mL in ethanol (NCBI, 2020d)

Vapour pressure: 1.92×10^{-7} mm Hg at 25 °C (estimated) (NCBI, 2020d); 6.80×10^{-7} mm Hg at 25 °C (estimated) (US EPA, 2020b)

Flash point: 222 °C (predicted) (US EPA, 2020b)

Octanol/water partition coefficient (P): log K_{ow} = 5.72 (estimated) (NCBI, 2020d)

Ultraviolet maximum: 262 nm (in chloroform) (NCBI, 2020d).

(d) Impurities

Leucomalachite green has a purity of ≥ 95%, with impurities of malachite green, monodesmethyl leucomalachite green, and 4-(dimethylamino) benzophenone (Culp et al., 1999; Le Goff & Wood, 2008; ECHA, 2010).

1.2 Production and use

1.2.1 Malachite green

(a) Production process

Malachite green is produced by condensing benzaldehyde and *N*,*N*-dimethylaniline in the molecular ratio 1 : 2 in the presence of sulfuric acid, zinc chloride, or oxalate salts. This is followed by further oxidation of the initial condensation product (leucomalachite green) with lead (IV) oxide or manganese (IV) oxide in the presence of hydrochloric acid. Novel processes, which are economical and environmentally acceptable, use catalytic oxidation with atmospheric oxygen or hydrogen peroxide. For dyeing purposes, malachite green is prepared as a double salt with zinc chloride, whereas for use in fish, zinc-free oxalate salts are used (NTIS, 1974; Gessner & Mayer, 2000; Agunwa & Okonkwo, 2004).

(b) Production volume

Because of its colour strength and brilliance, malachite green is one of the most economically important dyes. In 1993, approximately 9000 tonnes of basic di- and triphenylmethane dyes, including malachite green, were sold (Gessner & Mayer, 2000). In the USA, the production of malachite green chloride was > 0.454 tonnes in 1972 and 145 tonnes in 1975,

whereas the amount imported in the same years was 96 tonnes and 30.3 tonnes, respectively (NCBI, 2020b). In 2008 and 2011, malachite green was imported into Canada in quantities ranging from 1 to 100 tonnes (Health Canada, 2018). In 2020, malachite green chloride was available from 24 suppliers in China, 5 suppliers in the USA, and 2 suppliers in India (Chemical Register, 2020a). [Data on quantities produced and used elsewhere in the world were not available to the Working Group.]

(c) Uses

Malachite green is commonly used to dye a wide variety of materials including cotton, silk, wool, jute, leather, paper, inks, toners, waxes, and acrylic products (Sabnis, 2007). It is also used as a pigment in ceramics and in arts, crafts, and hobby materials, as well as in cosmetics including semipermanent hair dyes and body oils (Health Canada, 2018).

Malachite green is also used as a biological stain for the microscopic analysis of cells and tissues. As a primary stain, malachite green is used in the Schaeffer–Fulton staining technique to isolate endospores by staining them green (Schaeffer & Fulton, 1933). It can also be used as a counterstain in the Giménez staining method for *Rickettsia* species (Giménez, 1964) and *Helicobacter pylori* (Suvarna et al., 2013), as well as for determining acid-fast bacteria, mainly mycobacteria (Bueke et al., 1932), using the Ziehl–Neelsen method. Malachite green is also used in Alexander stain to discriminate aborted from non-aborted pollen, which is a method commonly applied in agriculture (Peterson et al., 2010).

Malachite green is also used as an analytical reagent in several assays, including: the quantification of released phosphate in the phosphate assay; the quantitative determination of cerium (IV) in silicate rocks, plant tissue, or water; and the determination of antimony (III) and antimony (V) in solution. Malachite green has been applied to microbial resistogram typing used to define the profile of a strain based on its resistance to selected compounds (Cooksey, 2016). It is used as pH indicator with a colour change from yellow at pH 0.0 to green at pH 2.0, and from green at pH 11.6 to colourless at pH 14.0 (Sabnis, 2007).

Finally, as a pharmacologically active substance, malachite green – typically malachite green oxalate – is used as a disinfectant in aquariums and for the farming of fish and shellfish. It has been the most effective agent known to treat water mould infections caused by *Saprolegnia* spp. in fish and eggs. It is also effective against protozoan ectoparasites, e.g. *Ichthyophthirius multifiliis*, and for the treatment of proliferative kidney disease when used as a bath or with prolonged immersion (Noga, 2010). In the past, malachite green was reported to be used as a fungicide or insecticide to treat seeds (NIOSH, 1973).

1.2.2 Leucomalachite green

(a) Production process

Leucomalachite green is produced by the condensation of benzaldehyde and *N*,*N*-dimethylaniline in the molecular ratio 1 : 2 in the presence of zinc chloride or oxalate salts (NTIS, 1974).

(b) Production volume

In 2020, leucomalachite green was available from 14 suppliers in China, 2 suppliers in the USA, and 1 supplier in India (Chemical Register, 2020b).

[Data on quantities produced were not available to the Working Group.]

(c) Uses

Leucomalachite green is used as a dye precursor to malachite green (Gessner & Mayer, 2000). Other uses of leucomalachite green include as a reagent in several analytical appli-

cations, e.g. for the quantitative colorimetric determination of haemoglobin and other haem compounds in forensic science. Haemoglobin catalyses a reaction between leucomalachite green and hydrogen peroxide, converting colourless leucomalachite green into malachite green, indicating the presence of blood (Slaunwhite et al., 1979). It is also used as a reagent for the spectrophotometric determination of arsenic (III) in environmental samples. Arsenic reacts with potassium iodate in acidic conditions to generate iodine, which oxidizes leucomalachite green to malachite green (Revanasiddappa et al., 2007).

Leucomalachite green is a component of radiochromic dosimeters that indicate exposure to radioactivity upon colour change (Alqathami et al., 2016).

1.3 Methods of detection and quantification

Representative methods for the detection and quantification of malachite green and leucomalachite green are summarized in Table 1.1.

1.3.1 Air

No methods for the detection and quantification of either malachite green or leucomalachite green in air were identified in the literature.

1.3.2 Water

There are several methods for the detection and quantification of malachite green in environmental and aquaculture water, as well as in wastewater samples (Table 1.1; summarized in Zhou et al., 2019). To quantify malachite green in water, ultraviolet-visible spectroscopy can be conducted; however, liquid chromatography (LC) combined with spectroscopy or mass spectrometry (MS) detection are more sensitive techniques (Tkaczyk et al., 2020). Due to the low concentrations of malachite green in natural waters, application of a pre-treatment step is required to concentrate the dye before analysis. Many techniques have been used for this purpose, including magnetic, ionic liquid, nanoparticle materials, solid-phase extraction, and microextraction techniques such as magnetic solid-phase extraction and dispersive liquid–liquid microextraction (Zhou et al., 2019). Most LC methods for the measurement of malachite green in water samples have a limit of detection between 0.01 and 0.1 µg/L.

1.3.3 Soil

Leucomalachite green has been determined in river sediment and soil samples obtained near a dye manufacturing plant using Soxhlet extraction with 2-propanol and gas chromatography-mass spectrometry (Nelson & Hites, 1980). Samples of river-suspended particulate matter and sediment influenced by municipal sewage effluents have been analysed for malachite green, and leucomalachite green, using extraction with acetonitrile and hydroxylamine and analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a detection capability of 1 µg/L (Weiß & Schmutzger, 2010; Table 1.1).

1.3.4 Food, beverages, and consumer products

Various methods for the determination and quantification of malachite green and leucomalachite green in food samples are detailed in the literature (Table 1.1; Hashimoto et al., 2011; Zhou et al., 2019). Most methods have been developed for residue analysis of the dyes in aquaculture products including fresh and processed fish, shrimp, and shellfish. However, residues of malachite green and leucomalachite green can also be measured in beef, pork, chicken, eggs, milk (Park et al., 2020), and Chinese softshell turtle (Shen et al., 2019). In fish treated with malachite

Table 1.1 Representative methods for the detection and quantification of malachite green and leucomalachite green in various matrices

Sample matrix	Sample preparation	Analytical technique	Agent	LOD (unless otherwise stated)	References
Water					
Wastewater (laundry, paper, printing, and textile effluent)	Lignocellulose biomass composite biosorbent SPE, evaporation, reconstitution in methanol/water, and filtration	UPLC-MS/MS	MG	0.1 µg/L 0.4 µg/L (LOQ)	Khan et al. (2019)
Water	NR	EESI-MS/MS	MG	0.5–3.8 µg/L	Fang et al. (2016)
Water	MCPE using Triton X-114	UV-vis spectrophotometry	MG	4.1 µg/L 13.6 µg/L (LOQ)	Ghasemi & Kaykhaii (2016)
Aquaculture water	Monolithic fibre SPME, evaporation, and reconstitution in methanol	HPLC-vis/FLD	MG LMG	0.05 µg/L 0.04 µg/L (LOQ) 0.05 µg/ 0.04 µg/L (LOQ)	Wang et al. (2015)
Water	TC-IL-DLLME using 1-octyl-3-methylimidazolium hexafluorophosphate	HPLC-UV–vis	MG	0.086 µg/L	Zhang et al. (2012)
Water	Maghemite nanoparticle-SPE	UV–vis spectrophotometry	Sum of MG + LMG	0.28 µg/L	Afkhami et al. (2010)
Water	Diol-SPE	LC-vis/FLD LC-MS/MS	MG LMG MG LMG	0.05 µg/L 0.04 µg/L 0.04 µg/L 0.03 µg/L	Mitrowska et al. (2008b)
Soil					
Suspended particulate matter and sediment	Extraction with ACN, HAH, and filtration	LC-MS/MS	MG LMG	1.8 µg/L 3.6 µg/L (LOQ) 1.6 µg/L 3.0 µg/L (LOQ)	Weiß & Schmutzger (2010)
River sediment and soil	Soxhlet extraction with 2-propanol	GC-MS	LMG	NR	Nelson & Hites (1980)
Food					
Beef, pork, chicken, egg, milk, flatfish, eel, and shrimp	Extraction with ACN/acetic acid, anhydrous sodium sulfate, purification with d-SPE using C_{18}, and PSA filtration	LC-MS/MS	MG, LMG	2 µg/kg (LOQ)	Park et al. (2020)
Trout and shrimp	Extraction with HAH, ACN/ascorbic acid, anhydrous magnesium sulfate, and heated ultrasonic treatment	LC-MS/MS	MG LMG	0.13 µg/L (CCα) 0.16 µg/L (CCβ) 0.18 µg/L (CCα) 0.24 µg/L (CCβ)	Eich et al. (2020)

Table 1.1 (continued)

Sample matrix	Sample preparation	Analytical technique	Agent	LOD (unless otherwise stated)	References
Trout, salmon, and prawns	Extraction with ACN, magnesium sulfate, filtration, oxidation with DDQ, evaporation, and reconstitution in ACN/ascorbic acid	LC-MS/MS	Sum of MG + LMG	0.04 μg/kg (CCα)	Dubreil et al. (2019)
Chinese softshell turtle (*Pelodiscus sinensis*)	Extraction with HAH, ammonium acetate buffer pH 4.5, ACN, evaporation, reconstitution in ACN, HLB-SPE, evaporation, and reconstitution in ACN	UPLC-MS/MS	MG LMG	0.16 μg/kg 0.52 μg/kg (LOQ) 0.18 μg/kg 0.60 μg/kg (LOQ)	Shen et al. (2019)
Fish blood and extracts	Extraction with ACN, alumina-SPE, and TiO_2 nanoflake dispersion	SALDI-TOF-MS	MG LMG	10 pg/mL 10 pg/mL	Gao et al. (2019)
Trout, salmon, catfish, tilapia, shrimp, Arctic char, barramundi, eel, frog legs, hybrid striped bass, pompano, scallops, sea bream, smoked trout, dried shrimp, and highly processed canned eel and dace products; the canned products contained oil, salt, sugar, flavourings, spices, sauces, and/or preservatives	Extraction with HAH, ACN, magnesium sulfate, evaporation, reconstitution in ACN/ascorbic acid, and filtration	LC-MS/MS	MG LMG	< 0.6 μg/kg 0.25 μg/L (CCα) 0.32 μg/L (CCβ) < 1.0 μg/kg (LOQ) 0.17 μg/kg (CCα) 0.22 μg/kg (CCβ)	Andersen et al. (2018) Hurtaud-Pessel et al. (2011)
Trout	Extraction with ACN and water, and filtration	HPLC-HR-TOF-MS	MG LMG	0.001 μg/kg 0.005 μg/kg (LOQ) 0.1 μg/kg 0.3 μg/kg (LOQ)	Amelin et al. (2017)
Shellfish (hard clam and oyster)	Extraction with ACN and n-hexane, filtration	LC-MS/MS	MG LMG	0.25–0.50 μg/kg (LOQ) 0.25–0.50 μg/kg (LOQ)	Chang et al. (2016)
Rainbow trout and sea bass	Extraction with ACN/acetic acid, evaporation, and reconstitution in ACN/acetic acid	LC-MS/MS	MG LMG	0.43 (CCα) μg/kg 0.56 (CCβ) μg/kg	Kaplan et al. (2014)

Table 1.1 (continued)

Sample matrix	Sample preparation	Analytical technique	Agent	LOD (unless otherwise stated)	References
Eel	Extraction with ACN, sodium acetate, oxidation with DDQ, evaporation, reconstitution in McIlvaine buffer pH 6.5/ACN, CBA and SCX-SPE, evaporation, reconstitution in ammonium acetate buffer pH 4.5/ACN, and filtration	LC-MS/MS	Sum of MG + LMG	< 0.01 µg/kg 0.25 µg/kg (LOQ)	Reyns et al. (2014)
Tilapia	QuEChERS using ACN/acetic acid, magnesium sulfate, sodium acetate, PSA, evaporation, reconstitution in ACN/ammonium acetate buffer pH 4/ascorbic acid, and filtration	LC-MS/MS	MG LMG	0.38 µg/kg (CCα) 0.55 µg/kg (CCβ 0.25 µg/kg (CCα) 0.39 µg/kg (CCβ)	Hashimoto et al. (2012)
Silver carp, crucian carp, tilapia, mandarin fish, and bream	Extraction with HAH/*p*-TSA/ammonium acetate/ACN, LLE with dichloromethane, diethylene glycol, ACN, evaporation, reconstitution in ACN, MCAX-SPE, evaporation, reconstitution in ammonium acetate/ACN/formic acid, and filtration	UPLC-MS	MG LMG	0.15 µg/kg 0.50 µg/kg (LOQ) 0.15 µg/kg 0.50 µg/kg (LOQ)	Xu et al. (2012)
Fish	Extraction with ammonium acetate buffer pH 4.5, ACN, d-SPE with alumina, LLE with dichloromethane, formic acid, oxidation with DDQ, and SCX-SPE	LC-MS/MS	Sum of MG + LMG	1.2 µg/kg (CCα) 2.0 µg/kg (CCβ)	Tarbin et al. (2008)
Trout, salmon, and shrimp	Extraction with ammonium acetate buffer pH 4.5, HAH, *p*-TSA, ACN, LLE with dichloromethane, evaporation, reconstitution in ACN, oxidation with DDQ, alumina- and propylsulfonic acid-SPE, evaporation, and reconstitution in ammonium acetate buffer pH 4.5/ACN	LC-vis LC-MS^n	Sum of MG + LMG	1.0 µg/kg 0.25 µg/kg	Andersen et al. (2009)
Salmon, rainbow trout, shrimp, and tilapia	Extraction with perchloric acid/ACN, dichloromethane, evaporation, C18-SPE, evaporation, and reconstitution in ACN	LC-MS/MS	MG LMG	0.1 µg/kg 0.1 µg/kg	van de Riet et al. (2005)

Table 1.1 (continued)

Sample matrix	Sample preparation	Analytical technique	Agent	LOD (unless otherwise stated)	References
Carp	Extraction with HAH, acetate buffer pH 4.5, *p*-TSA, ACN, LLE with dichloromethane, SCX-SPE, evaporation, reconstitution in ACN/acetate buffer pH 4.5/ascorbic acid	LC-vis/FLD	MG LMG	0.15 µg/L (CCα) 0.37 µg/L (CCβ) 0.13 µg/kg (CCα) 0.32 µg/kg (CCβ)	Mitrowska et al. (2005)
Catfish, eel, rainbow trout, salmon, tropical prawns, and turbot	Extraction with McIlvaine buffer pH 3.0, *p*-TSA, methanolic TMPD, ACN, McIlvaine buffer pH 6, LLE with dichloromethane, aromatic sulfonic acid-bonded-SPE, evaporation, reconstitution in sample-solvent, post-column oxidation with PbO_2	HPLC-vis LC-MS/MS	MG, LMG (as MG)	1.0 µg/kg 0.2 µg/kg	Bergwerff & Scherpenisse (2003)

ACN, acetonitrile; C18, octadecyl; CBA, cation exchange cartridges; CCα, decision limit: the concentration level at which there is probability α (usually defined as 1% for non-authorized substances) that a blank sample will give a signal at this level or higher; CCβ, detection capability: the concentration level at which there is a probability β (usually defined as 5%) that the method will give a result lower than CCα; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; d-SPE, dispersive solid-phase extraction; EESI, extractive electrospray ionization; HAH, hydroxylamine hydrochloride; HLB, hydrophilic–lipophilic balance; HPLC, high-performance liquid chromatography; HR-TOF, high-resolution quadrupole time-of-flight; GC, gas chromatography; LC, liquid chromatography; LLE, liquid–liquid extraction; LMG, leucomalachite green; LOD, limit of detection; LOQ, limit of quantification; MCAX, C8 and cation exchange compound cartridge; MCPE, micro-cloud point extraction; MG, malachite green; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MS^n, multiple-stage mass spectrometry; NR, not reported; PbO_2, lead dioxide; PSA, primary secondary amine; *p*-TSA, *para*-toluenesulfonic acid; QuEchERS, quick easy cheap effective rugged safe; SALDI-TOF, surface-assisted laser desorption/ionization time-of-flight mass; SCX, strong cation exchange; SPE, solid-phase extraction; SPME, solid-phase microextraction; TC-IL-DLLME, temperature-controlled ionic liquid dispersive liquid–liquid microextraction; TiO_2, titanium dioxide; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine dihydrochloride; UPLC, ultra-performance liquid chromatography; UV, ultraviolet; vis, visible light; vis/FLD, visible light and fluorescence detection.

green, residues of malachite green and leucomalachite green may be detected in muscle at up to 56 days and up to 252 days, respectively, after the end of exposure. Due to its long residence time in tissues, leucomalachite green is the marker residue for the monitoring of malachite green usage in aquaculture products (Mitrowska et al., 2008a). Therefore, methods must permit residue analysis of both the chromatic dye and its colourless leuco form (Verdon & Andersen, 2017). The primary analytical methods for the detection of malachite green and leucomalachite green consist of LC separation-based methods. They are usually designed to measure both substances separately using a combination of visible detection (618 nm) for the chromatic dye and fluorescence detection (λ_{ex}, 265 nm; and λ_{em}, 360 nm) for the colourless leuco form, or after column oxidation of leucomalachite green to its parent form using lead oxide or iodine. Another option is to measure both substances as malachite green following oxidation of leucomalachite green to its parent form using 2,3-dichloro-5,6-dicyanobenzoquinone (then measured by visible detection) or as leucomalachite green after the reduction of malachite green to its leuco form with potassium borohydride (then measured by fluorescence detection). Thanks to its identification and confirmation capabilities, LC-MS/MS is the method of choice for confirmatory analysis of both substances in food, although other MS detectors such as ion trap and time-of-flight have also been used, all providing limit of detection values that are typically below 1 µg/kg. The most commonly used pre-treatment protocol applied before instrumental detection of target analytes is performed by treating extracted muscle samples with an acidic buffer and acetonitrile, liquid–liquid partitioning, and solid-phase extraction. However, faster extraction techniques with greater selectivity, including molecularly imprinted solid-phase extraction and the quick easy cheap effective rugged safe (QuEChERS) technique, have also been proposed (Hashimoto et al., 2012).

1.3.5 Biological specimens

No methods for the detection and quantification of either malachite green or leucomalachite green in human blood, urine, or saliva were identified in the literature. [The Working Group noted that the methods used for fish described in Section 1.3.4 could be useful for analysing material from humans or experimental animals.]

1.4 Occurrence and exposure

1.4.1 Environmental occurrence

Malachite green is not known to occur naturally in the environment. The major sources of environmental release of malachite green are the chemical manufacturing plants where it is produced, factories where it is used, and release from cosmetic products such as hair dye and other products such as dyed clothing and coloured papers (Health Canada, 2020a). Considering its physicochemical properties, if released into the air, malachite green will exist solely in the particulate phase. This phase is removed from the atmosphere by wet and dry deposition. As a cationic dye, it will be adsorbed more strongly to organic carbon and clay than its neutral counterparts. Volatilization from moist or dry soil surfaces is not expected to be important. Biodegradation is not expected to be an important fate process in the environment (NCBI, 2020b). Malachite green is most likely to be found in industrial wastewater (Khan et al., 2019) and is expected to adsorb to suspended solids and sediments based upon its cationic form. [The Working Group noted that the solubility of malachite green in water is several orders of magnitude higher than that of leucomalachite green and that the octanol/water partition coefficient of malachite green is one order of magnitude

higher, which has implications for its fate in the environment.] A study performed in Germany (Ricking et al., 2013) detected malachite green in suspended particulate matter from several German rivers, as well as in sediment cores from the Spree and Havel rivers in the urban area of Berlin. Malachite green, but not its leuco form, was detected at increasing concentrations of up to 543 µg/kg. Under anaerobic conditions, malachite green is known to be transformed into its corresponding leuco compound via a reversible reaction (Weiß & Schmutzger, 2010; Ricking et al., 2013). However, one study found malachite green to be the primary sedimentary pollutant under both natural and anaerobic conditions (Ricking et al., 2013). In the USA, 11 aromatic amines related to the commercial production of malachite green and gentian violet were found in soil and sediment from a bank of the Buffalo River, New York, close to a dyestuff manufacturing plant (Nelson & Hites, 1980).

Malachite green exists almost entirely in its ionized form and volatilization from water surfaces is not expected to be an important fate process. In China, a study reported limited data on the presence of malachite green in natural waters (Zhang et al., 2012). Theoretical estimations of concentrations of non-sulfonated triarylmethane dyes in surface water, which also represented drinking-water, were calculated for three industrial sources in Canada based on the maximum production capacities of these industries: 3.2×10^{-4} mg/L from the paper-dyeing industry, 9.5×10^{-4} mg/L from the de-inking industry, and 2.1×10^{-4} mg/L from the general formulation industry. These conservative estimates were made for gentian violet, malachite green, and two other triarylmethane dyes, assuming that any one of the non-sulfonated triarylmethane dyes could be substituted for another (Health Canada, 2020a). Malachite green may undergo hydrolysis and photolysis reactions under environmental conditions to form demethylated, hydroxylated, and benzophenone products (Mitrowska et al., 2008b; Pérez-Estrada et al., 2008). In one of these studies, the reduction of malachite green to leucomalachite green in water was also observed (Pérez-Estrada et al., 2008). However, this was not observed in another study (Mitrowska et al., 2008b). Many research activities worldwide are focused on improving the treatment of wastewater from the dye industry through biological, chemical, and physical processes (Shindhal et al., 2021).

An estimated bioconcentration factor of 3 suggests that the potential for bioconcentration in aquatic organisms is low. For triarylmethane dyes, partitioning to proteins in cell membranes is more likely to occur than partitioning to lipids (Health Canada, 2020a). A study conducted in Germany in 2007 measured residues of malachite green (expressed as the sum of malachite green and leucomalachite green) in tissue samples taken from wild-living eels caught in surface waters (lakes and rivers) that contained treated sewage effluents (Schuetze et al., 2008). The residue concentrations ranged from 0.051 to 0.346 µg/kg depending on the sampling location. A similar study, conducted in Belgium, analysed 16 dyes including triarylmethanes and their metabolites in muscle samples taken from individual yellow-phased European eels (*Anguilla anguilla*) between 2000 and 2009 from 91 locations in rivers, canals, and lakes (Belpaire et al., 2015). Malachite green and leucomalachite green were detected in samples from 41.8% and 26.4% of the locations, respectively. The sum of malachite green and leucomalachite green detected ranged from 0.12 to 9.96 µg/kg.

1.4.2 Occurrence in food

Malachite green can be used as a veterinary drug for the treatment of disease in fish and shellfish. In animals treated with malachite green, the major metabolite (leucomalachite green) has a longer residence time in fatty muscle than its parent compound, thus leucomalachite

green is considered the marker residue in the monitoring of malachite green usage in aquaculture (Mitrowska et al., 2008a). [The Working Group noted that in the reports described below, methods either detected malachite green and leucomalachite green separately or detected total residues as the sum of malachite green plus leucomalachite green after leucomalachite green was oxidized to malachite green.] Several papers have reported the presence of malachite green and leucomalachite green in wild and farmed fish from different countries in Europe, Asia, and North America, with different analytical methods used to detect and confirm the presence of the agents. Among the reports, the maximum concentrations observed ranged from 0.9 to 146 µg/kg (Table 1.2). According to reports from the European Food Safety Authority (EFSA; 2015–2020), several Member States reported samples that were non-compliant for the presence of malachite green, leucomalachite green, or their sum, in their national veterinary drug residue control plan. In the European Rapid Alert System for Food and Feed, the peak of notifications of non-compliant samples (50 samples) was reported in 2005 and, since 2008, fewer than 10 notifications per year have been reported, which have been ascribed to imports or trade between Member States (European Commission, 2020). A study conducted in India reported the occurrence of non-permitted colourants in food, including sweets, hard-boiled sugar confectionery, beverages, bakery items, savouries, ice-candy, ice-cream, crushed ice, sugar toys, and miscellaneous food commodities. Malachite green was detected in 1.25% of the 1199 foodstuffs analysed, with higher concentrations in edible samples collected from rural markets than those from urban markets. The authors of this study speculated that the findings may reflect adulteration or improper usage through ignorance due to the low cost and easy accessibility of malachite green (Tripathi et al., 2007).

1.4.3 Occupational exposure

No contemporary occupational exposure information was found for malachite green or leucomalachite green. [The Working Group noted that occupational exposure to malachite green and leucomalachite green may occur through dermal contact and inhalation at workplaces where the compound is produced or applied, as described in Sections 1.1.2 and 1.2.2.] In the 1970s, the use of fungicides and pesticides (including malachite green) was investigated at a seed-manufacturing company in California, USA (NIOSH, 1973). A malachite green slurry was used to coat seeds, and low-level exposure of workers to malachite green while operating the coating machine and malachite green dust in the bagging area was reported.

A survey of occupations and industries was conducted between 1981 and 1983 by the National Institute for Occupational Safety and Health in the USA (NIOSH, 2017). This survey estimated that 181 763 workers were potentially exposed to malachite green chloride, with 44% listed as machinists, 21% as machine operators, 4% as janitors and cleaners, and 3% in medical or scientific occupations. [The Working Group noted that it is unclear whether these percentages reflect modern exposure patterns, given the age of the study.]

1.4.4 Exposure in the general population

In the general population, exposure can occur through contact with textiles, paper, and inks containing malachite green, the occasional treatment of diseased ornamental tropical fish with malachite green, the use of hair dye containing malachite green, and the consumption of fish, shellfish, or drinking-water containing residues of malachite green and leucomalachite green. In an EFSA report on malachite green in food, an EFSA panel on contaminants in the food chain concluded that available occurrence data were

Table 1.2 Detection and quantification of malachite green and leucomalachite green in aquaculture products available on the international market

Country reported	Agent	Year	Analytical method	Sample type	No. of samples tested	No. of positive samples (> LOD)	Concentration (µg/kg)		Reference
							Mean	Range	
Belgium	MG	2000–2009	LC-MS/MS	Eel[a]	91	23	NR	< 0.05–0.96	Belpaire et al. (2015)
	LMG					38	0.56	< 0.05–9.61	
Netherlands	LMG	NR	LC-vis and LC-MS/MS	Trout	18	13	3.2[b]	< 1–14.9	Bergwerff & Scherpenisse (2003)
	LMG			Eel	10	5	4.5[b]	< 1–9.7	
	LMG			Fresh, smoked, or canned salmon	20	5	0.7[b]	< 0.2–2.9	
Armenia	MG + LMG	2017	ELISA and LC-MS/MS	Sevan trout	11	8	1.1	0.3–3	Pipoyan et al. (2020)
	MG + LMG			Rainbow trout	16	12	2.1	0.3–4.8	
	MG + LMG			Sturgeon	2	2	2.5	2.5[c]	
Canada	LMG	2018	LC-MS/MS	Fish and shellfish	56	7	NR	< 0.003–0.9	Dinh et al. (2020)
Malaysia	MG + LMG	2013	LC-MS/MS	Fish (five species, imported and local)	37	17	NR	0.53–4.10	Kwan et al. (2018)
Iran (Islamic Republic of)	MG	2014–2015	CEI	Carp and trout	177	108	1.6	< 0.3–7.12	Barani & Tajik (2017)
Iran (Islamic Republic)	MG	2011	LC-vis	Trout	144	70	5.89	< 0.3–146.1	Fallah & Barani (2014)
Croatia	MG	2009–2011	Immunoassay	Carp and trout	72	2	0.231	< 0.1–1.07	Bilandžić et al. (2012)
Canada	LMG	1993–2004	LC-MS/MS	Marine fish, freshwater fish, and shrimp	39	3	0.96[b]	0.73–1.20	Tittlemier et al. (2007)

CEI, competitive immunoassay; ELISA, enzyme-linked immunosorbent assay; LC, liquid chromatography; LMG, leucomalachite green; LOD, limit of detection; MG, malachite green; MS/MS, tandem mass spectrometry; NR, not reported; vis, visible light.

[a] Eels were not for human consumption in this study.

[b] Means calculated from concentrations in samples in which the compound was detected.

[c] Identical values.

not suitable for a reliable exposure assessment. Based on a reference point for action of 2 µg/kg for the sum of malachite green and leucomalachite green as an occurrence value for all types of fish, fish products, and crustaceans, mean dietary exposure was calculated across different European dietary surveys and age classes. Exposure would range from 0.1 to 5 ng/kg body weight (bw) per day. For high-quantity and frequent fish consumers, the exposure would range from 1.3 to 11.8 ng/kg bw per day (EFSA CONTAM Panel, 2016). A screening assessment performed by Health Canada considered the use of hair dyes and drinking-water consumption to be the main routes for exposure to malachite green. For hair dye use, a potential daily dose of 0.0102 mg/kg bw per day for adults was estimated. For drinking-water, a potential daily dose of 0.0001 mg/kg bw per day was estimated based on predicted theoretical environmental concentrations in surface water because of environmental release by the paper de-inking industry. Other exposure scenarios considered, but not taken into account in the estimation because of lower estimated exposures, were surface-water levels due to the industrial release from paper dyeing in mills and production facilities, consumer "down-the-drain" releases, exposure via food, and the use of other consumer products containing malachite green as a pigment, such as paper products, mixtures, and other manufactured items (Health Canada, 2020b).

1.5 Regulations and guidelines

1.5.1 Exposure limits and guidelines

Malachite green chloride is very toxic to aquatic life (acute H400 and chronic H410), is harmful if swallowed (H302), causes serious eye damage (H318), and is suspected of damaging the fetus (H361d) (ECHA, 2020c).

The Joint Food and Agriculture Organization of the United Nations/World Health Organization Expert Committee on Food Additives (JECFA) has not established an acceptable daily intake for malachite green or its metabolite leucomalachite green, and has not supported the use of malachite green for food-producing animals, thus no maximum residue limits for malachite green and leucomalachite green have been recommended (WHO, 2009a).

Malachite green is not registered for use in food-producing animals in the European Union, UK, Canada, USA, Australia, New Zealand, Brazil, or Chile (Verdon & Andersen, 2017). Malachite green is not permitted as a food additive in Canada (Health Canada, 2018). Malachite green and leucomalachite green are not permitted as food additives or in food packaging in the USA (US FDA, 2020, 2021). Malachite green has also been prohibited in cosmetics by the European Commission (European Commission, 2009), in Canada (Health Canada, 2018; US FDA, 2021), Australia and New Zealand (NZ EPA, 2019), and by the Association of Southeast Asian Nations (HSA, 2020). In food products derived from animals for which malachite green use is prohibited, there is a zero-tolerance concentration for residues of malachite green and/or its metabolite leucomalachite green, the marker residue indicating use of malachite green (WHO, 2009a). Depending on the country, regulatory limits from 0.5 to 2.0 µg/kg for malachite green and leucomalachite green, or for the sum of the residues, are used in national and international residue monitoring programmes (Verdon & Andersen, 2017).

Leucomalachite green is suspected of causing genetic defects (H341) and is suspected of causing cancer (H351) (ECHA, 2020d). No regulations were found for leucomalachite green.

1.5.2 Reference values for biological monitoring of exposure

No reference values for biological monitoring of malachite green or leucomalachite green exposure in humans were found.

2. Cancer in Humans

No data were available to the Working Group.

3. Cancer in Experimental Animals

3.1 Malachite green

See Table 3.1.

3.1.1 Mouse

Oral administration (feed)

In a study of chronic toxicity and carcinogenicity that complied with Good Laboratory Practice (GLP) and that was conducted by the National Toxicology Program (NTP) (NTP, 2005) and published as Culp et al. (2006), four groups of 48 female B6C3F$_1$/Nctr Br (C57BL/6N × C3H/HeN MTV$^-$) mice (age, approximately 6 weeks) were given feed containing malachite green chloride (purity, 87%; impurities were identified as leucomalachite green, 7.5%; *N*-desmethyl malachite green, 3.8%; and *N*-desmethyl leucomalachite green, 0.5%; malachite green chloride also contained 1.4% methanol by weight) at a concentration of 0, 100, 225, or 450 ppm (representing average daily doses of 0, 15, 33, and 67 mg/kg bw per day, respectively), for the control group and the groups at the lowest, intermediate, and highest dose, respectively, for 104 weeks. Owing to limitations on the number of groups that could be included and the fact that females were more sensitive than males to the toxicity of malachite green chloride in the dose-finding studies, the 2-year study was restricted to female mice only. Throughout the study, no significant difference in survival was observed between groups treated with malachite green chloride and controls. Survival was 40/48, 44/48, 40/48, and 41/48 for the control group and groups at the lowest, intermediate, and highest dose, respectively. Mean body weight and feed consumption of the female mice treated with malachite green were similar to those of control mice. Complete necropsies and full histopathological examination were performed.

No treatment-related neoplasms were observed in female mice treated with malachite green chloride. [The Working Group noted that this was a well-conducted study that complied with GLP, that the duration of exposure and observation was adequate, and that multiple doses and large numbers of mice per group were used, but males were not included.]

3.1.2 Rat

(a) Oral administration (feed)

In a study that complied with GLP and that was conducted by the NTP (2005), and published as Culp et al. (2006), four groups of 48 female F344/N Nctr Br rats (age, approximately 6 weeks) were given feed containing malachite green chloride (purity, 87%; impurities were identified as leucomalachite green, 7.5%; *N*-desmethyl malachite green, 3.8%; and *N*-desmethyl leucomalachite green, 0.5%; malachite green chloride also contained 1.4% methanol by weight) at a concentration of 0, 100, 300, or 600 ppm (representing average daily doses of 0, 7, 21, and 43 mg/kg bw per day, respectively), for the control group and the groups at the lowest, intermediate, and highest dose, respectively, for 104 weeks. Similar to the above study in mice, owing to limitations on the number of groups that could be included and the fact that female rats were more sensitive than males to the toxicity of malachite green chloride in the dose-finding studies, the 2-year study

Table 3.1 Studies of carcinogenicity with malachite green and leucomalachite green in experimental animals

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$/Nctr Br (C57BL/6N × C3H/HeNMTV$^-$) (F) ~6 wk 104 wk NTP (2005), Culp et al. (2006)	Oral MG chloride, 87%; impurities: LMG (7.5%), *N*-desmethyl MG (3.8%), and *N*-desmethyl LMG (0.5%); 1.4% methanol by weight Feed 0, 100, 225, 450 ppm (equivalent to 0, 15, 33, 67 mg/kg bw per day) 48, 48, 48, 48 40, 44, 40, 41	No significant increase in tumour incidence in treated animals		Principal strengths: complied with GLP; multiple-dose study; high number of mice per group; adequate duration of exposure and observation Principal limitations: only one sex tested Other comments: relative kidney weights of exposed groups of mice were generally lower than those of the controls
Full carcinogenicity Rat, F344/N Nctr Br (F) ~6 wk 104 wk NTP (2005), Culp et al. (2006)	Oral MG chloride, 87%; impurities: LMG (7.5%), *N*-desmethyl MG (3.8%), and *N*-desmethyl LMG (0.5%); 1.4% methanol by weight Feed 0, 100, 300, 600 ppm (equivalent to 0, 7, 21, 43 mg/kg bw per day) 48, 48, 48, 48 29, 23, 32, 25	*Thyroid gland* Follicular cell adenoma 0/46, 0/48, 1/47, 1/46 Follicular cell carcinoma 0/46, 0/48, 2/47, 1/46 Follicular cell adenoma or carcinoma (combined) 0/46, 0/48, 3/47 (6%)*, 2/46 (4%)	 NS NS $P = 0.032$, poly-3 trend test adjusted for differences in body weight; *$P = 0.035$, poly-3 pairwise test adjusted for differences in body weight (Culp et al., 2006); $P = 0.064$ (NS), poly-3 trend test (NTP, 2005)	Principal strengths: complied with GLP; multiple-dose study; high number of rats per group; adequate duration of exposure and observation Principal limitations: only one sex tested Other comments: mean body weights of female mice at 300 and 600 ppm were generally lower than those of the controls; relative liver weights were significantly increased in the group of female mice treated at 600 ppm. Incidence in historical controls: thyroid follicular cell adenoma or carcinoma (combined) 7/517 (1.4%) (range, 0–3%), no thyroid follicular cell carcinoma reported, hepatocellular adenoma 1/541 (0.2%) (range, 0–0.6%), mammary gland carcinoma 4/534 (0.7%) (range, 0–4%), and pituitary gland adenoma or carcinoma (combined) 306/528 (58.0%) (range, 51–68%)

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Rat, F344/N Nctr Br (F) ~6 wk 104 wk NTP (2005), Culp et al. (2006) (cont.)		*Liver*: hepatocellular adenoma		
		1/48 (2%), 1/48 (2%), 3/48 (6%), 4/48 (8%)*	*P* = 0.048, poly-3 trend test adjusted for differences in body weight; **P* = 0.006, poly-3 pairwise test adjusted for differences in body weight (Culp et al., 2006); *P* = 0.059 (NS), poly-3 trend test (NTP, 2005)	
		Mammary gland: carcinoma		
		2/48 [2/46], 2/48 (4%), 1/48 (2%), 5/48 (10%)	*P* = 0.011, poly-3 trend test adjusted for differences in body weight (Culp et al., 2006); *P* = 0.113, poly-3 trend test (NTP, 2005). [NS], Cochran–Armitage trend test (using 2/46 at 0 ppm)	
		Pituitary gland (pars distalis): adenoma		
		26/48 (54%), 36/47 (77%)*, 32/46 (70%), 29/45 (64%)	**P* = 0.014, poly-3 pairwise test (NTP, 2005)	
Full carcinogenicity Mouse, B6C3F$_1$/Nctr Br (C57BL/6N × C3H/HeNMTV$^-$) (F) ~6 wk 104 wk NTP (2005), Culp et al. (2006)	Oral LMG, 99% Feed 0, 91, 204, 408 ppm (equivalent to 0, [13], 31, 63 mg/kg bw per day) 48, 48, 48, 48 37, 41, 39, 39	*Liver* Hepatocellular adenoma		Principal strengths: complied with GLP; multiple-dose study; high number of mice per group; adequate duration of exposure and observation Principal limitations: only one sex tested Other comments: relative kidney weights were significantly decreased in all treated groups Incidence in historical controls: hepatocellular adenoma, 26/563 (4.6%) (range, 0–11%); hepatocellular carcinoma, 8/563 (1.4%) (range, 0–4%); hepatocellular adenoma or carcinoma (combined), 34/563 (6.0%) (range, 0–11%)
		3/47 (6%), 6/48 (12%), 5/47 (10%), 9/47 (18%)	NS	
		Hepatocellular carcinoma		
		0/47, 0/48, 1/47 (2%), 2/47 (4%)	NS	
		Hepatocellular adenoma or carcinoma (combined)		
		3/47 (6%), 6/48 (13%), 6/47 (13%), 11/47 (23%)*	*P* = 0.013, poly-3 trend test; **P* = 0.022, poly-3 pairwise test (NTP, 2005); *P* = 0.002, poly-3 trend test adjusted for differences in body weight; **P* = 0.004, poly-3 pairwise test adjusted for differences in body weight (Culp et al., 2006)	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Rat, F344/N Nctr Br (M) ~6 wk 104 wk NTP (2005), Culp et al. (2006)	Oral LMG, 99% Feed 0, 91, 272, 543 ppm (equivalent to 0, 5, 15, 30 mg/kg bw per day) 48, 47, 48, 47 23, 29, 34, 30	*Thyroid gland* Follicular cell adenoma 0/47, 2/47, 0/48, 1/46 Follicular cell carcinoma 0/47, 0/47, 1/48, 2/46 Follicular cell adenoma or carcinoma (combined) 0/47, 2/47 (4%), 1/48 (2%), 3/46 (6%) *Testis* Interstitial cell adenoma, bilateral 22/48, 30/47, 38/48, 39/47* Interstitial cell adenoma (including bilateral) 37/48 (77%), 42/47 (89%) (*), 43/48 (90%) (**), 45/47 (96%)*, (***) *Liver*: hepatocellular adenoma 2/48, 2/47, 3/48, 2/47	NS NS NS *$P < 0.05$, poly-3 pairwise test (NTP, 2005) $P = 0.036$, poly-3 trend test; *$P = 0.029$, poly-3 pairwise test (NTP, 2005); $P = 0.001$, poly-3 trend test adjusted for differences in body weight; (*) $P = 0.009$, (**) $P = 0.008$, (***) $P = 0.001$, poly-3 pairwise test adjusted for differences in body weight (Culp et al., 2006) NS	Principal strengths: complied with GLP; multiple-dose study; high number of rats per group; adequate duration of exposure and observation; used males and females Other comments: survival of rats treated at 272 ppm was greater than that of the controls; mean body weights of rats treated at 543 ppm were lower than those of the controls throughout the study; mean body weights of rats treated at 272 ppm were lower than those of the controls during year 2 of the study; feed consumption by rats treated at 543 ppm was intermittently less than that of controls throughout the study; liver weights were significantly increased for rats treated at 272 and 543 ppm; relative thyroid gland weights of rats treated at 543 ppm were significantly increased Historical controls: thyroid follicular cell adenoma or carcinoma (combined), 2/511 (0.4%) (range, 0–2%); no thyroid follicular cell carcinoma reported; interstitial cell adenoma of the testis, 469/547 (85.7%) (range, 69–90%)

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Rat, F344/N Nctr Br (F) ~6 wk 104 wk NTP (2005), Culp et al. (2006)	Oral LMG, 99% Feed 0, 91, 272, 543 ppm (equivalent to 0, 6, 17, 35 mg/kg bw per day) 48, 48, 48, 48 33, 36, 35, 33	*Thyroid gland* Follicular cell adenoma 0/46, 0/46, 0/47, 1/48 Follicular cell carcinoma 0/46, 1/46, 2/47, 0/48 Follicular cell adenoma or carcinoma (combined) 0/46, 1/46 (2%), 2/47 (4%), 1/48 (2%) *Mammary gland* Adenoma 0/48, 1/48 (2%), 1/48 (2%), 2/48 (4%) Carcinoma 0/48, 1/48 (2%), 2/48 (4%), 2/48 (4%)	NS NS NS NS NS	Principal strengths: complied with GLP; multiple-dose study; high number of rats per group; adequate duration of exposure and observation; used males and females Other comments: mean body weights of rats treated at 543 and 272 ppm were lower than those of the controls throughout the study; mean body weights of rats treated at 91 ppm were lower than those of the controls during year 2 of the study; feed consumption by rats treated at 543 ppm was intermittently less than that of the controls throughout the study; feed consumption by rats treated at 272 ppm was intermittently lower during year 2 of the study; relative liver weights were significantly increased for rats treated at 272 and 543 ppm; relative thyroid gland weights of rats treated at 543 ppm were significantly increased Incidence in historical controls: thyroid follicular cell adenoma or carcinoma (combined), 7/517 (1.4%) (range, 0–3%); mammary gland adenoma or carcinoma (combined), 9/534 (1.7%) (range, 0–6%); mammary gland adenoma, 5/534 (0.9%) (range, 0–2%); mammary gland carcinoma, 4/534 (0.7%) (range, 0–4%)

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Rat, F344/N Nctr Br (F) ~6 wk 104 wk NTP (2005), Culp et al. (2006) (cont.)		Adenoma or carcinoma (combined) 0/48, 2/48 [2/46], 3/48 (6%)*, 4/48 (8%)	$P = 0.047$, poly-3 trend test (NTP, 2005); $P = 0.11$ (NS), poly-3 trend test adjusted for differences in body weight; $^*P = 0.008$, poly-3 pairwise test adjusted for differences in body weight (Culp et al., 2006); [NS], Cochran–Armitage trend test (using 2/46 at 91 ppm)	
		Liver: hepatocellular adenoma 1/48, 3/48, 0/48, 3/48	NS	

bw, body weight; F, female; GLP, Good Laboratory Practice; LMG, leucomalachite green; M, male; MG, malachite green; NS, not significant; ppm, parts per million; wk, week.

was restricted to female rats only. Throughout the study, no significant difference in survival was observed between groups treated with malachite green chloride and controls. Survival was 29/48, 23/48, 32/48, and 25/48 for the control group and the groups at the lowest, intermediate, and highest dose, respectively. There were significant decreases in mean body weight in the groups at the intermediate and highest dose compared with controls. Throughout the study, feed consumption by treated female rats was generally similar to that of controls. Complete necropsies and full histopathological examination were performed.

In female rats, there was a significant positive trend in the incidence of follicular cell adenoma or carcinoma (combined) of the thyroid gland (P = 0.032, body weight-corrected poly-3 trend test reported by Culp et al., 2006; and P = 0.064, poly-3 trend test reported by the NTP, 2005), with the incidence being significantly increased at the intermediate dose (P = 0.035, body weight-corrected poly-3 pairwise test reported by Culp et al., 2006). Two rats at the intermediate dose and one at the highest dose developed follicular cell carcinoma of the thyroid gland. In addition, follicular cell hyperplasia of the thyroid gland, a preneoplastic lesion, was only observed in female rats at the intermediate and highest dose, and a significant positive trend was reported in the incidence of cystic follicles in the thyroid gland, a non-neoplastic lesion. [The Working Group considered the occurrence of follicular cell adenoma or carcinoma (combined) of the thyroid gland to be treatment-related; this was supported by the low incidence of these tumours in the historical controls, 7/517 female Fischer 344 rats (1.4%; range, 0–3%), which developed only follicular cell adenomas of the thyroid gland.] There was a significant positive trend in the incidence of hepatocellular adenoma (P = 0.048, body weight-corrected poly-3 trend test reported by Culp et al., 2006; and P = 0.059, poly-3 trend test reported by the NTP, 2005), with the incidence being significantly increased at the highest dose compared with controls (P = 0.006, body weight-corrected poly-3 pairwise test, as reported by Culp et al., 2006) and exceeding the upper bound of the range observed in historical controls in this laboratory (1/541 rats; range, 0–0.6%). At the intermediate dose, a single hepatocellular carcinoma was found in a female rat that also had an adenoma. The incidence of eosinophilic foci, a preneoplastic liver lesion, was also significantly increased compared with controls, and centrilobular necrosis was only observed in female rats at the highest dose. Mammary gland carcinomas were observed in all groups of female rats: control, 2/48 [2/46]; lowest dose, 2/48 (4%); intermediate dose, 1/48 (2%); and highest dose, 5/48 (10%)). Culp et al. (2006) reported a significant positive trend (P = 0.011, body weight-corrected poly-3 trend test), while the NTP (2005) reported no statistically significant increases. In addition, the incidence in the group at the highest dose exceeded the upper bound of the range observed in historical controls (4/534; range, 0–4%) in this laboratory. [The Working Group noted that the NTP (2005) gave the denominators as the numbers of animals necropsied, but that two female controls with missing mammary glands were included. If the number of animals with mammary glands examined microscopically was presented using tumour data for individual animals from Table B2a on page 125 of the report from the NTP (2005), the incidence would be 2/46, 2/48, 1/48, and 5/48 for the control group and groups at the lowest, intermediate, and highest dose, respectively, which would weaken the outcome of the trend test. Since it was impossible to replicate the trend test performed by Culp et al. (2006) because of the corrections made by Gaylor & Kodell (2001), the Working Group could only conclude that the development of mammary gland carcinomas may have been related to treatment.] There was a significant increase in the incidence of adenoma of the pituitary gland (pars distalis) at the lowest

dose (P = 0.014, poly-3 pairwise test reported by the NTP, 2005) compared with controls: control, 26/48 (54%); lowest dose, 36/47 (77%); intermediate dose, 32/46 (70%); and highest dose, 29/45 (64%). In addition, incidence in the groups at the lowest and intermediate dose exceeded the upper bound of the range observed in historical controls (pituitary gland [pars distalis] neoplasms, 306/528; range, 51–68%) in this laboratory. [The Working Group noted that this was a well-conducted study that complied with GLP, that the duration of exposure and observation was adequate, and multiple doses and large numbers of rats per group were used, but that males were not included.]

(b) Oral administration (gavage)

In a study by Werth & Unnewehr (1966), two groups of 13 pairs of albino rats [age and strain not reported] were treated with malachite green by gavage [purity, dose, and dosing regimen not reported] or with malachite green by gavage [purity, dose, and dosing regimen not reported] followed by an intravenous dose of cytochrome c [purity, dose, and dosing regimen not reported]. A third group, consisting of a number [not reported] of pairs of rats, was untreated and served as parents for the control group. The offspring of parent rats of all three groups were untreated. Ten generations of rats (about 5000 rats) were followed up, and there were approximately 2000 offspring of the group of rats treated with malachite green only; it was reported that many of these offspring died at an early age, whereas adverse effects were rarely observed in the offspring of the two other groups.

Histopathological examination was performed, and no tumours were observed in the parent generations of all three groups. Across 10 generations, no tumours were observed in the control group or in the offspring of rats treated with malachite green plus cytochrome c. Tumours were observed in 57 out of about 2000 offspring of rats treated with malachite green only: mammary gland tumours (mainly fibroadenomas and carcinomas) and lung tumours (mainly carcinomas) were observed in 19/57 and 13/57 tumour-bearing rats, respectively. No malignant tumours had been reported in historical controls from the laboratory. [The Working Group noted the unusual study design and the unclear and incomplete reporting. The study was considered inadequate for the evaluation of the carcinogenicity of malachite green in experimental animals due to its limitations and is not tabulated or considered further.]

3.2 Leucomalachite green

See Table 3.1.

3.2.1 Mouse

Oral administration (feed)

In a study that complied with GLP and that was conducted by the NTP (2005) and published as Culp et al. (2006), four groups of 48 female B6C3F$_1$/Nctr Br (C57BL/6N × C3H/HeN MTV$^-$) mice (age, approximately 6 weeks) were given feed containing leucomalachite green (purity, 99%) at a concentration of 0, 91, 204, or 408 ppm (representing average daily doses of 0, [13], 31, or 63 mg/kg bw per day, respectively) for the control group and groups at the lowest, intermediate, and highest dose, respectively, for 104 weeks. Owing to limitations on the number of groups that could be included and the observation that female mice were more sensitive than males to the toxicity of leucomalachite green in the dose-finding studies, the 2-year study was restricted to female mice only. Throughout the study, no significant difference in survival was observed between groups treated with leucomalachite green and controls. Survival was 37/48, 41/48, 39/48, and 39/48 for the control groups and groups at the lowest, intermediate, and highest dose, respectively. Throughout the study, mean body weights of the female mice treated

with leucomalachite green were similar to those of controls. There was no significant difference in feed consumption between the treated groups and controls. Complete necropsies and full histopathological examinations were performed.

In female mice, there was a significant positive trend in the incidence of hepatocellular adenoma or carcinoma (combined) (P = 0.013, poly-3 trend test, reported by the NTP, 2005; and P = 0.002, body weight-corrected poly-3 trend test, reported by Culp et al., 2006) with the incidence being significantly increased at the highest dose (11/47, 23%; P = 0.022, poly-3 pairwise test, reported by the NTP, 2005; and P = 0.004, body weight-corrected poly-3 pairwise test reported by Culp et al., 2006). The incidence of hepatocellular adenoma or carcinoma (combined) in all groups treated with leucomalachite green exceeded the upper bound of the range observed in historical controls (34/563; range, 0–11%) in this laboratory. [The Working Group noted that this was a well-conducted study that complied with GLP, the duration of exposure and observation was adequate, and multiple doses and large numbers of mice per group were used, but that males were not included.]

3.2.2 Rat

Oral administration (feed)

In a study that complied with GLP and that was conducted by the NTP (2005) and published as Culp et al. (2006), four groups of 47–48 male and 48 female F344/N Nctr Br rats (age, approximately 6 weeks) were given feed containing leucomalachite green (purity, 99%) at a concentration of 0, 91, 272, or 543 ppm (representing average daily doses of 0, 5, 15, or 30 mg/kg bw per day for males, and 0, 6, 17, and 35 mg/kg bw per day for females, respectively) for the control group and groups at the lowest, intermediate, and highest dose, respectively, for 104 weeks. The dose range-finding study with leucomalachite green was conducted using males only; however, because female rats appeared to be more sensitive than males to the toxicity of malachite green chloride, both sexes were included in the 2-year bioassay with leucomalachite green (NTP, 2005). Survival of males and females treated with leucomalachite green was similar to that of their respective controls, except that the survival of males at the intermediate dose was greater than that of controls (control, 23/48; intermediate dose, 34/48). In males, the mean body weight of the group at the highest dose was lower than that of the controls throughout the study, and the mean body weight of the group at the intermediate dose was lower than that of the controls during the second year of the study. In females, the mean body weights of the groups at the intermediate and highest dose were lower than those of the control group throughout the study, and the mean body weight of the group at the lowest dose was lower than that of the controls during the second year of the study. Feed consumption was intermittently lower in males and females at the highest dose than in the respective controls throughout the study, and in females at the intermediate dose during the second year of the study. Complete necropsies and full histopathological examinations were performed.

In male rats, there was an increase in the incidence of follicular cell adenoma or carcinoma (combined) of the thyroid gland (control, 0/47; lowest dose, 2/47 (4%); intermediate dose, 1/48 (2%); and highest dose, 3/46 (6%)). Although the increase did not reach statistical significance, it was noted that incidence at the highest dose exceeded the upper bound of the range observed in historical controls in this laboratory (2/511; range, 0–2%). In addition, one rat at the intermediate dose and two at the highest dose developed follicular cell carcinoma of the thyroid gland, whereas these tumours were not observed in 511 historical controls. [The Working Group considered the occurrence of follicular cell adenoma or carcinoma (combined) of the thyroid gland to be related to treatment.] In addition, there

was a significant positive trend in the incidence of testicular interstitial cell adenoma (including bilateral): control, 37/48 (77%); lowest dose, 42/47 (89%); intermediate dose, 43/48 (90%); and highest dose, 45/47 (96%) ($P = 0.036$, poly-3 trend test reported by the NTP, 2005; $P = 0.001$, body weight-corrected poly-3 trend test, reported by Culp et al., 2006). The report by the NTP (2005) noted that incidence at the highest dose was significantly higher than in the control group ($P = 0.029$, poly-3 pairwise test), while the statistical analysis by Culp et al. (2006) found that incidence in each of the groups treated with leucomalachite green was significantly higher than in the control group ($P = 0.009$, $P = 0.008$, and $P = 0.001$ for the lowest, intermediate, and highest dose, respectively; body weight-corrected poly-3 pairwise test). In addition, the incidence of interstitial cell adenoma (including bilateral) of the testis at the highest dose exceeded the upper bound of the range observed in historical controls in this laboratory (469/547; range, 69–90%). There was a high incidence of bilateral interstitial cell adenoma of the testis in rats in this group that were removed early in the study due to morbidity or death. The NTP (2005) also reported a significant increase in the incidence of bilateral interstitial cell adenoma of the testis at the highest dose compared with the control group ($P < 0.05$, poly-3 pairwise test).

In female rats, there was a significant positive trend in the incidence of adenoma or carcinoma (combined) of the mammary gland (control, 0/48; lowest dose, 2/48 [2/46]; intermediate dose, 3/48 (6%); and highest dose, 4/48 (8%); $P = 0.047$, poly-3 trend test; reported by the NTP, 2005; $P = 0.11$, body weight-corrected poly-3 trend test, reported by Culp et al., 2006), with incidence at the highest dose exceeding the upper bound of the range observed in historical controls in this laboratory (9/534; range, 0–6%). Culp et al. (2006) reported that incidence at the intermediate dose was significantly higher than that in the control group ($P = 0.008$, body weight-corrected poly-3 pairwise comparison). [The Working Group noted that the report by the NTP (2005) gave the denominators as the number of rats necropsied, but that two females at the lowest dose with missing mammary glands were included. If the number of rats with mammary glands examined microscopically were presented using tumour data for individual animals from Table B2b on page 155 of the report by the NTP (2005), the incidence would be as follows: control, 0/48; lowest dose, 2/46; intermediate dose, 3/48; and highest dose, 4/48, which would modify the outcome of the trend test. Since it was impossible to replicate the trend test performed by NTP (2005) and Culp et al. (2006) because of the corrections made by Gaylor & Kodell (2001), the Working Group considered that the mammary gland tumours were related to treatment on the basis of the significant increase in incidence at the intermediate dose and because these tumours are uncommon in this strain of rat, but noted the uncertainty of the trend.] There was a marginal increase in the incidence of follicular cell adenoma or carcinoma (combined) of the thyroid gland: 0/46, 1/46 (2%), 2/47 (4%), and 1/48 (2%), respectively. Although not statistically significant, the incidence of follicular cell adenoma or carcinoma (combined) of the thyroid gland at the intermediate dose exceeded the upper bound of the range observed in historical controls in this laboratory (7/517; range, 0–3%). [The Working Group noted that that this was a well-conducted study that complied with GLP, that multiple doses, a large number of rats per group, and males and females were used, and that the duration of exposure and observation was adequate.]

3.3 Evidence synthesis for cancer in experimental animals

3.3.1 Malachite green

The carcinogenicity of malachite green has been assessed in one study in female mice and one study in female rats exposed to malachite green chloride by oral administration (in the feed), and in a multigeneration study in offspring of rats exposed to malachite green by oral administration (gavage).

In a study that complied with GLP (NTP, 2005; Culp et al., 2006), female B6C3F$_1$/Nctr Br (C57BL/6N × C3H/HeN MTV$^-$) mice were given feed containing malachite green chloride. No treatment-related neoplasms were observed.

In a study that complied with GLP (NTP, 2005; Culp et al., 2006), female F344/N Nctr Br rats were given feed containing malachite green chloride. There was a significant positive trend and significant increase in the incidence of hepatocellular adenoma and of follicular cell adenoma or carcinoma (combined) of the thyroid gland. There was a significant increase in the incidence of adenoma of the pituitary gland (pars distalis).

In a multigeneration study by Werth & Unnewehr (1966), rats were treated with malachite green by gavage, and followed for 10 generations without further treatment. [The study was considered inadequate for the evaluation of the carcinogenicity of malachite green in experimental animals.]

3.3.2 Leucomalachite green

The carcinogenicity of leucomalachite green has been assessed in one study in female mice and one study in male and female rats exposed by oral administration (in the feed).

In a study that complied with GLP (NTP, 2005; Culp et al., 2006), female B6C3F$_1$/Nctr Br (C57BL/6N × C3H/HeN MTV$^-$) mice were given feed containing leucomalachite green. There was a significant positive trend and significant increase in the incidence of hepatocellular adenoma or carcinoma (combined).

In a study that complied with GLP (NTP, 2005; Culp et al., 2006), male and female F344/N Nctr Br rats were given feed containing leucomalachite green. In males, there was a treatment-related increase in the incidence of follicular cell adenoma or carcinoma (combined) of the thyroid gland, and a significant positive trend and significant increase in the incidence of testicular interstitial cell adenoma. In females, there was a significant increase in the incidence of adenoma or carcinoma (combined) of the mammary gland.

4. Mechanistic Evidence

4.1 Absorption, distribution, metabolism, and excretion

4.1.1 Humans

No studies on the absorption, distribution, metabolism, or excretion of malachite green or leucomalachite green in exposed humans were available to the Working Group. Severe methaemoglobinaemia (51%) was observed in a girl aged 3 years who incidentally ingested a commercially available aquarium product containing 45 mg of malachite green (Spiller et al., 2008).

One study in vitro showed that human intestinal microflora from faecal samples was able to reduce malachite green to leucomalachite green almost completely (99%) and that 14 cultures of anaerobic bacteria species representative of those found in the human gastrointestinal tract were able to convert 7.3–99% of malachite green to its reduced form (Henderson et al., 1997).

4.1.2 Experimental systems

See Fig. 4.1.

In male and female rats given [^{14}C]-labelled malachite green as an oral dose at 2 mg/kg bw, 96 ± 6% of the administered dose was excreted in the faeces and urine over 7 days, with faeces accounting for 80% of the cumulative excretion of radiolabel. Tissue distribution was not investigated due to the low levels of blood and tissue radiolabel (US FDA, 1994; reviewed in NTP, 2005; and WHO, 2009b).

The nature and the quantities of metabolites of malachite green and leucomalachite green were investigated in liver extracts in a short-term exposure study in rats and mice given feed containing either malachite green or leucomalachite green (Culp et al., 1999). In liver extracts of Fischer 344 rats exposed to feed containing malachite green, mono-, di-, tri-, and tetradesmethyl malachite green derivatives and malachite green *N*-oxide were identified by atmospheric pressure chemical ionization–mass spectrometry (APCI-MS) analysis. A small, but measurable, amount of leucomalachite green was also detected. In liver extracts of rats exposed to leucomalachite green, mono-, di-, tri-, and tetradesmethyl leucomalachite green, malachite green *N*-oxide, desmethyl malachite green *N*-oxide, and didesmethyl malachite green *N*-oxide were also identified (Culp et al., 1999). In the liver of rats given feed containing leucomalachite green, qualitative analysis by high-performance liquid chromatography (HPLC) with ultraviolet detection showed that unmetabolized compound was the major product, accompanied by small amounts of mono- and didesmethyl leucomalachite green. In the liver of mice given feed containing malachite green, mono- and didesmethyl malachite green were detected, but no desmethyl leucomalachite green was identified (Culp et al., 1999).

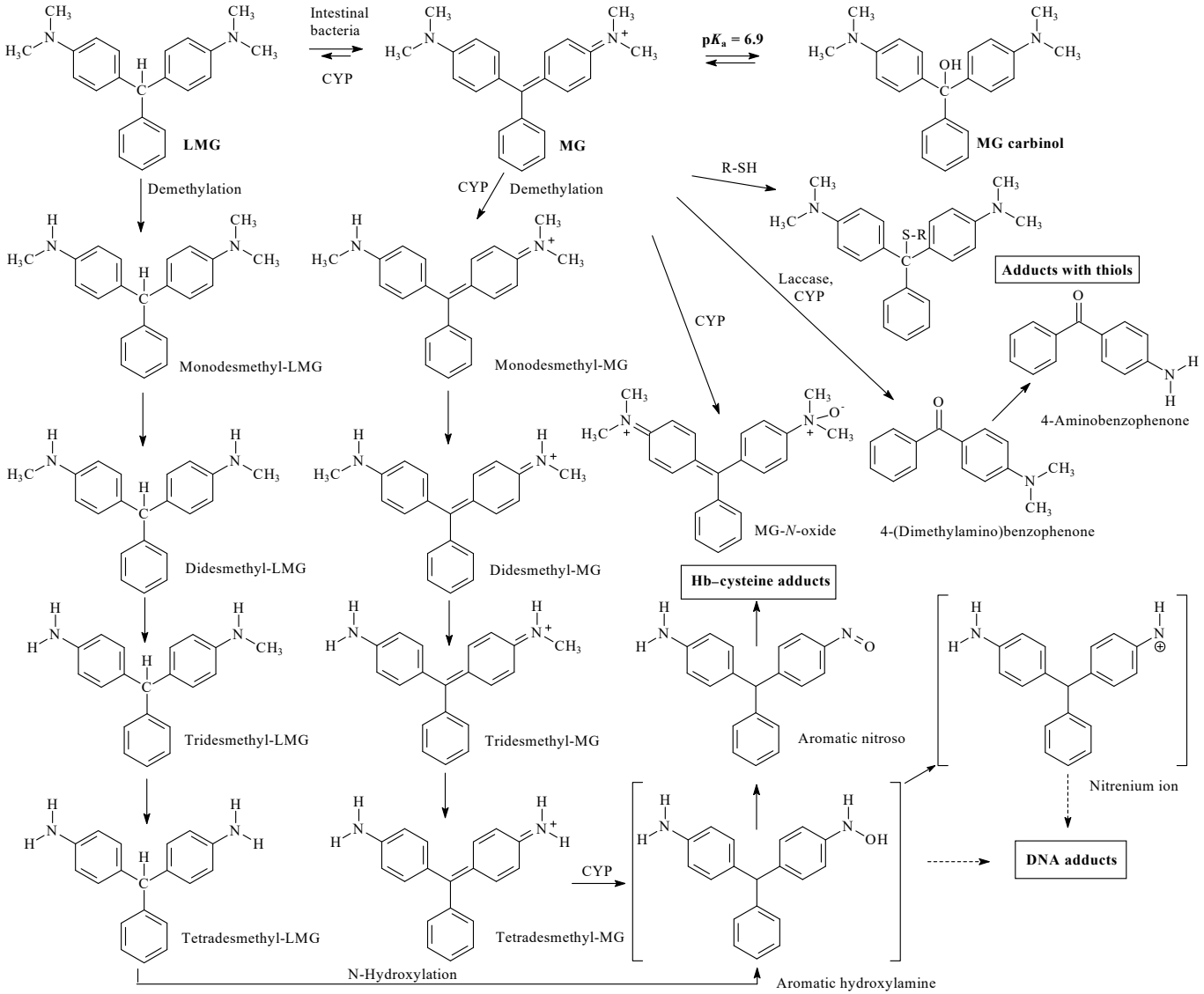
After intravenous injection of malachite green in rats, leucomalachite green was detected in the liver, kidney, heart, lung, and muscle after 2 hours (reviewed in NTP, 2005), which suggests that malachite green can be reduced to leucomalachite green in rat tissues (Werth & Boiteux, 1968). In cultures of intestinal bacteria from rats, mice, and rhesus monkeys under anaerobic conditions, malachite green was readily converted into leucomalachite green almost completely (99–100%) (Henderson et al., 1997). Singh et al. (1994) showed that malachite green was transformed by the faecal microflora of rats into one fluorescent metabolite. A study on biliary excretion in rats indicated that malachite green was extensively excreted via the bile, probably as a glutathione (GSH) adduct, reaching peak excretion 20 minutes after dosing (Debnam et al., 1993).

The metabolites desmethyl leucomalachite green, didesmethyl leucomalachite green, tridesmethyl leucomalachite green, malachite green, and malachite green *N*-oxide were also identified by online LC-APCI-MS in an in vitro incubation study on leucomalachite green with thyroid peroxidase (TPO), iodide, and tyrosine in the presence of an H_2O_2-generating system, which yielded oxidation products (Doerge et al., 1998; see Fig. 4.1). [The Working Group noted that information about the relative amounts of the different metabolites, including leucomalachite green, was sparse.]

4.2 Evidence relevant to key characteristics of carcinogens

This section summarizes the evidence for the key characteristics of carcinogens (Smith et al., 2016) for malachite green and leucomalachite green, including whether each agent is electrophilic or can be metabolically activated to an electrophile; is genotoxic; induces oxidative stress; modulates receptor-mediated effects; and causes immortalization. For the evaluation of other key characteristics of carcinogens, insufficient data were available.

Fig. 4.1 Proposed metabolic and bioactivation pathways for malachite green and leucomalachite green



CYP, cytochrome P450; Hb, haemoglobin; LMG, leucomalachite green; MG, malachite green; pK_a, negative log base 10 of the K_a value (acid dissociation constant); R-SH, thiol.
Arrows with solid lines indicate observed pathways; arrows with dashed lines indicated proposed pathways.
Created by the Working Group.

4.2.1 Is electrophilic or can be metabolically activated to an electrophile

Using monitoring of the bleaching of malachite green colour in the presence of human plasma, Tacal & Özer (2004) reported the cationic malachite green has the ability to form protein adducts. Furthermore, malachite green and leucomalachite green can be metabolized by demethylation to produce secondary or primary aromatic amines (Culp et al., 1999; Cha et al., 2001; Wang et al., 2012). These aromatic amines may be further metabolized to form aromatic nitrenium ions, which are highly electrophilic and can form adducts (IARC, 2021). DNA adducts have been observed in mammalian animals exposed to malachite green (Culp et al., 1999, 2002) (see details in Section 4.2.2).

4.2.2 Is genotoxic

Table 4.1, Table 4.2, and Table 4.3 summarize the available studies on the genetic and related effects of malachite green and leucomalachite green.

(a) Humans

No information on genotoxicity in exposed humans was available to the Working Group.

In one study in vitro, a concentration-dependent increase in DNA damage as measured by the comet assay was observed in THP-1 human monocytes exposed to malachite green, with the lowest effective concentration being 200 μM (Xiao et al., 2016).

(b) Experimental systems

(i) Non-human mammals in vivo

See Table 4.1.

Several studies investigated the genotoxic effects of exposure to malachite green or leucomalachite green in experimental animals in vivo. The end-points included DNA adducts, DNA damage, gene mutation, chromosomal aberration, micronucleus formation, and sister-chromatid exchange.

DNA adducts

DNA adducts, analysed by ^{32}P-postlabelling, were detected in the livers of male Fischer 344 rats and female B6C3F$_1$ mice given feed containing malachite green (Culp et al., 1999).

The formation of DNA adducts was also observed in the livers of rats exposed to leucomalachite green in the feed, but not in those of mice treated with the same dose (Culp et al., 1999). In rats, the response was stronger with malachite green (220 fmol adduct/mg DNA) than with leucomalachite green (180 fmol adduct/mg DNA). In another study, Culp et al. (2002) confirmed that formation of DNA adducts was observed in the livers of female Big Blue rats exposed to leucomalachite green. Moreover, the DNA adducts from Big Blue rats co-eluted with those from the livers of male Fischer 344 rats exposed to leucomalachite green in the feed (Culp et al., 2002). [The Working Group noted that the chemical structures and properties of these DNA adducts were not characterized.]

DNA damage

Malachite green-induced DNA damage was reported in several in vivo studies in mice. Dose-dependent DNA fragmentation (measured by the diphenylamine method) was observed in hepatocytes from male Swiss mice treated with malachite green by gavage (Donya et al., 2012). Significant induction of DNA damage (measured by the comet assay) was observed in lymphocytes in female Swiss albino mice treated with malachite green by intraperitoneal injection. Intake of the selenium compound diphenylmethyl selenocyanate (DMSE) can significantly attenuate the levels of DNA damage caused by malachite green (Das et al., 2013). Kasem et al. (2016) reported that significant DNA damage, measured by the comet assay, was seen in the livers of male mice (strains not specified) exposed orally to malachite green.

Table 4.1 Genetic and related effects of malachite green[a] and leucomalachite green in non-human mammals in vivo

End-point	Species, strain (sex)	Tissue	Results[b]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Malachite green							
DNA adducts (^{32}P-postlabelling assay)	Rat, F344 (M)	Liver	+	100 ppm	Feed, 28 days, 0, 100, or 600 ppm	Malachite green chloride	Culp et al. (1999)
DNA adducts (^{32}P-postlabelling assay)	Mouse, B6C3F$_1$ (F)	Liver	+	600 ppm	Feed, 28 days, 0, 100, or 600 ppm	Malachite green chloride	Culp et al. (1999)
DNA fragmentation (DPA assay)	Mouse, Swiss albino (M)	Liver	+	27 mg/kg bw per day	Gavage, 28 days, 27, 91, 272, or 543 mg/kg bw per day	Purity, NR	Donya et al. (2012)
DNA strand breaks (comet assay)	Mouse, Swiss albino (F)	Liver	+	4 mg/kg bw per day	Intraperitoneal injection, 30 days, 100 µg/mouse (25 g) bw	The control group did not receive intraperitoneal injection of solvent	Das et al. (2013)
DNA strand breaks (comet assay)	Mouse, NR (M)	Liver	+	2.5 mg/kg bw per day	Orally, 14 and 28 days, 0, 2.5, or 5 mg/kg bw per day	Analytical grade	Kasem et al. (2016)
Gene mutation (mouse spot test)	Mouse, C57B1/6J Han (F, pregnant)	Offspring	–	40 mg/kg bw per day	Gavage, 10, 20 and 40 mg/kg bw per day at days 8, 9, and 10 in pregnancy	Technical grade	Jensen (1984)
Gene mutation, *Hprt*	Mouse, Big Blue B6C3F$_1$ (F)	Spleen, lymphocyte	–	450 ppm/kg	Feed, 4 or 16 wk, 0 or 450 ppm	Malachite green chloride; purity, 88%	Mittelstaedt et al. (2004); NTP (2005)
Gene mutation, *cII*	Mouse, Big Blue B6C3F$_1$ (F)	Liver	–	450 ppm	Feed, 4 or 16 wk, 0 or 450 ppm	Malachite green chloride; purity, 88%	Mittelstaedt et al. (2004); NTP (2005)
Chromosomal aberrations	Mouse, Swiss albino (M)	Bone marrow	+	27 mg/kg bw per day (for 14, 21, or 28 days)	Gavage, 7, 14, 21, or 28 days, 27, 91, 272, or 543 mg/kg bw	Purity, NR	Donya et al. (2012)
Chromosomal aberrations	Mouse, Swiss albino (M)	Spermatocytes	+	27 mg/kg bw per day (for 21 or 28 days)	Gavage, 7, 14, 21, or 28 days, 27, 91, 272, or 543 mg/kg bw	Purity, NR	Donya et al. (2012)
Chromosomal aberrations	Mouse, Swiss albino (F)	Bone marrow	+	4 mg/kg bw per day	Intraperitoneal injection, 30 days, 100 µg/mouse (25 g) bw	One dose only; the control group did not receive intraperitoneal injection of solvent; purity, NR	Das et al. (2013)

Table 4.1 (continued)

End-point	Species, strain (sex)	Tissue	Results[b]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Micronucleus formation	Mouse, Swiss albino (F)	Bone marrow	+	4 mg/kg bw per day	Intraperitoneal injection, 30 days, 100 µg/mouse (25 g) bw	One dose only; the control group did not receive intraperitoneal injection of solvent; purity, NR	Das et al. (2013)
Micronucleus formation	Mouse, Big Blue B6C3F$_1$ (F)	Blood erythrocytes	–	450 ppm	Feed, 4 or 16 wk, 0 or 450 ppm	Malachite green chloride; purity, 88%	Culp & NTP (2004); Mittelstaedt et al. (2004)
Micronucleus formation	Mouse, NMRI:BOM (NR)	Bone marrow	–	37.5 mg/kg bw	Gavage, 24, 42, or 66 h, 37.5 mg/kg bw	Malachite green oxalate	Clemmensen et al., 1984
Micronucleus formation	Mouse, B6C3F$_1$ (M, F)	Blood erythrocytes	–	1200 ppm	Feed, 28 days, 25, 100, 300, 600, or 1200 ppm	Purity, 88%	Culp & NTP (2004)
Micronucleus formation	Rat, F344 (M)	Bone marrow	–	8.75 mg/kg bw	Intraperitoneal injection; 3×; sampled after 24 h, at 1.094, 2.188, 4.375, or 8.75 mg/kg bw	A small but significant increase was seen at the intermediate dose of 4.375 mg/kg, but not at 8.75 mg/kg bw; purity, 88%	Culp & NTP (2004)
Sister-chromatid exchange	Mouse, Swiss albino (M)	Bone marrow	+	91 mg/kg bw per day (for 21 or 28 days)	Gavage, 7, 14, 21, or 28 days, 27, 91, 272, or 543 mg/kg bw per day	Purity, NR	Donya et al. (2012)
Leucomalachite green							
DNA adducts (^{32}P-postlabelling assay)	Rat, F344 (M)	Liver	+	580 ppm	Feed, 28 days, 0, 96, or 580 ppm		Culp et al. (1999)
DNA adducts (^{32}P-postlabelling assay)	Mouse, B6C3F$_1$ (F)	Liver	–	580 ppm	Feed, 28 days, 0, 96, or 580 ppm		Culp et al. (1999)
DNA adducts (^{32}P-postlabelling assay)	Rat, Big Blue (F)	Liver	+	91 ppm	Feed, 4 wk, 0, 9, 27, 91, 272, or 543 ppm		Culp et al. (2002)
Gene mutation, *Hprt*	Rat, Big Blue (F)	Spleen, lymphocyte	–	543 ppm	Feed, 4, 16, or 32 wk, 0, 9, 27, 91, 272, or 543 ppm		Manjanatha et al. (2004)
Gene mutation, *Hprt*	Mouse, Big Blue B6C3F$_1$ (F)	Spleen, lymphocyte	–	408 ppm/kg	Feed, 4 or 16 wk; 0, 204, or 408 ppm		Mittelstaedt et al. (2004)
Gene mutation, *lacI*	Rat, Big Blue (F)	Liver	+	543 mg/kg	Feed, 4, 16, or 32 wk, 0, 9, 27, 91, 272, or 543 ppm	Positive at 16 wk only	Culp et al. (2002)

Table 4.1 (continued)

End-point	Species, strain (sex)	Tissue	Results[b]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Gene mutation, *lacI*	Rat, Big Blue (F)	Liver	–	543 ppm	Feed, 16 wk, 0 or 543 ppm	Reanalysed by clonally corrected *lacI* mutation frequency	Manjanatha et al. (2004)
Gene mutation, *cII*	Mouse, Big Blue B6C3F$_1$ (F)	Liver	+	408 ppm	Feed, 4 or 16 wk, 0, 204, or 408 ppm		Culp & NTP (2004); Mittelstaedt et al. (2004)
Micronucleus formation	Rat, Big Blue (F)	Bone marrow	–	543 ppm	Feed, 4, 16, or 32 wk, 0, 9, 27, 91, 272, or 543 ppm		Manjanatha et al. (2004)
Micronucleus formation	Rat, F344 (M)	Bone marrow	–	8.75 mg/kg bw	Intraperitoneal injection, 3×, sampled after 24 h; at 1.094, 2.188, 4.375, 8.75 mg/kg bw		Culp & NTP (2004)
Micronucleus formation	Mouse, B6C3F$_1$ (M, F)	Blood erythrocytes	–	1160 ppm	Feed; 28 ×; sampled after 24 h, at 25, 100, 300, 600, 1200 ppm		Culp & NTP (2004)
Micronucleus formation	Mouse, Big Blue B6C3F$_1$ (F)	Blood erythrocytes	–	408 ppm	Feed, 4 or 16 wk, 0, 204, or 408 ppm		Culp & NTP (2004); Mittelstaedt et al. (2004)

bw, body weight; DPA, colorimetric determination by diphenylamine; F, female; h, hour; HID, highest ineffective dose; *Hprt*, hypoxanthine-guanine phosphoribosyltransferase; LED, lowest effective dose; M, male; NR, not reported; ppm, parts per million; wk, week.

[a] Except where noted, the form of the agent that was tested was not specified.

[b] +, positive; –, negative.

Table 4.2 Genetic and related effects of malachite green[a] and leucomalachite green in non-human mammalian cells in vitro

End-point	Species, tissue, cell line	Results[b]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Malachite green						
DNA strand breaks (comet assay)	Chinese hamster, ovary, CHO-K1 cells	+		3 µg/mL	Oxalate form; purity, 70.8%; > 3 µg/mL cytotoxic	Fessard et al. (1999)
DNA strand breaks (comet assay)	Chinese hamster, ovary, CHO-K1 cells		+	15 µg/mL	Oxalate form; purity, 70.8%	Fessard et al. (1999)
DNA strand breaks (alkaline elution)	Syrian hamster, embryo, SHE cells	+	NT	1 µg/mL	Purity, NR	Panandiker et al. (1994)
DNA strand breaks (alkaline elution)	Syrian hamster, embryo, SHE cells	+	NT	1 µg/mL	Purity, NR	Mahudawala et al. (1999)
DNA strand breaks (comet assay)	Syrian hamster, embryo, SHE cells	+	NT	0.025 µg/mL	Purity, NR	Bose et al. (2005)
DNA strand breaks (comet assay)	Syrian hamster, embryo, SHE cells	+	NT	0.1 µg/mL	Purity, NR	Ashra & Rao (2006)
DNA strand breaks (comet assay)	Transformed Syrian hamster, embryo, cells	+	NT	0.1 µg/mL	Purity, NR	Ashra & Rao (2006)
Intercalation (DNA binding assay)	Cow, thymus, DNA	+	NT	20 ng/mL	Malachite green chloride, purity, NR	Cheng & Li (2009)
Gene mutation, *Hprt*	Chinese hamster, ovary, CHO-K1 cells	(+)		0.01 µg/mL	Oxalate form; purity, 70.8% (not reproducible; no dose-related response)	Fessard et al. (1999)
Gene mutation, *Hprt*	Chinese hamster, ovary, CHO-K1 cells		(+)	0.1 µg/mL	Oxalate form; purity, 70.8% (not reproducible; no dose-related response)	Fessard et al. (1999)
Chromosomal aberrations	Chinese hamster, ovary, CHO cells	–	NT	20 µM	Purity, NR	Au & Hsu (1979)
Chromosomal abnormalities (flow cytometry and chromosomal pattern)	Syrian hamster, embryo, SHE cells	+	NT	0.025 µg/mL	Purity, NR	Mahudawala et al. (1999)
Leucomalachite green						
DNA strand breaks (comet assay)	Chinese hamster, ovary, CHO-K1 cells	–		500 µg/mL	Purity, NR	Fessard et al. (1999)
DNA strand breaks (comet assay)	Chinese hamster, ovary, CHO-K1 cells		–	300 µg/mL	Purity, NR	Fessard et al. (1999)

Table 4.2 (continued)

End-point	Species, tissue, cell line	Results[b]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Gene mutation, *Hprt*	Chinese hamster, ovary, CHO-K1 cells	(+)		75 µg/mL	Purity, NR; no dose-related response	Fessard et al. (1999)
Gene mutation, *Hprt*	Chinese hamster, ovary, CHO-K1 cells		(+)	5 µg/mL	Purity, NR; positive in only one trial; dose-related response	Fessard et al. (1999)

HIC, highest ineffective concentration; *Hprt*, hypoxanthine-guanine phosphoribosyltransferase; LEC, lowest effective concentration; NR, not reported; NT, not tested.

[a] Except where noted, the form of the agent that was tested was not specified.

[b] +, positive; –, negative; (+), positive in a study of limited quality.

Table 4.3 Genetic and related effects of malachite green[a] and leucomalachite green in non-mammalian experimental systems

Test system (species, strain)	End-point	Results[b]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Malachite green						
Immature pea pods	DNA-intercalating, induction of phenylalanine ammonia lyase and pisatin synthesis	+	NA	1.0 mg/mL	Purity, NR	Hadwiger & Schwochau (1971)
Fish, *Hemichromis bimaculatus,* blood	DNA damage (comet assay)	+	NA	0.75 µg/mL	Purity, NR	Souza et al. (2020)
Fish, *Channa striata*, kidney cell line	DNA damage (comet assay)	+	NT	0.1 µg/mL	Purity, NR	Majeed et al. (2014)
Fish, *Channa striata*, gill cell line	DNA damage (comet assay)	+	NT	0.1 µg/mL	Purity, NR	Majeed et al. (2014)
Green algae, *Chlorella pyrenoidosa*	DNA damage (measured by RAPD analysis and DAPI staining)	+	NA	1.75 µM	Malachite green oxalate; purity, NR	Kanhere et al. (2014)
Bacillus subtilis, NIG17 rec^+ and NIG45 rec^-	DNA damage (rec assay)	–	NT	3 µg/well	Purity, NR; R50 = 1.1 (ratio of 50% survival concentrations)	Matsui (1980)
Trout eggs	Chromosomal aberrations	+	NA	Unspecified	Malachite green oxalate; purity, NR	Lieder (1961)
Chironomid larvae	Chromosomal derangement	+	NA	Unspecified	Purity, NR	Keyl & Werth (1959)
Drosophila larvae	Chromosomal derangement	+	NA	100 ppm	Malachite green chloride; purity, NR	Pfeiffer (1961) (in German)
Allium cepa	Chromosome and nuclear aberrations	+	NA	122.66 mg/L	Purity, NR; a non-significant effect on micronuclei and chromosome breaks was reported	Shanmugam et al. (2017)
Allium cepa	Chromosome and nuclear aberrations	–	NA	122.66 mg/L	Mixture after laccase metabolism	Shanmugam et al. (2017)
Green algae, *Chlorella pyrenoidosa*	Chromosomal aberrations (DAPI fluorescence staining)	+	NA	1.75 µM	Malachite green oxalate; purity, NR	Kanhere et al. (2014)
Fish, *Hemichromis bimaculatus*, blood	Micronucleus formation	–	NA	0.75 µg/mL	Purity, NR	Souza et al. (2020)

Table 4.3 (continued)

Test system (species, strain)	End-point	Results[b]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Fish, common carp	Micronucleus formation in blood	–	NA	0.5 mg/L	Water bath, at concentration of 0.5 mg/L for 6 days	Svobodová et al. (1997)
Baker's yeast (Fleischmann)	Mutation, respiration-deficient (*petite colonie*)	+	NT	1.0 µg/mL	Purity, NR	Nagai (1959)
Salmonella typhimurium, TA98	Reverse mutation	–	+	30 µg/plate	Malachite green oxalate	Clemmensen et al. (1984)
Salmonella typhimurium, TA98	Reverse mutation	NT	+	75 µg/plate	Analytical grade	Ayed et al. (2017)
Salmonella typhimurium, TA98	Reverse mutation	NT	–	75 µg/plate	Mixture after biodegradation with *Staphylococcus aureus*	Ayed et al. (2017)
Salmonella typhimurium, TA98, TA100	Reverse mutation	–	NT	500 µg/plate	Malachite green oxalate; before and after biodegradation	Cheriaa et al. (2012)
Salmonella typhimurium, TA98, TA97, TA1537	Reverse mutation	–	NT	Unspecified	Purity, NR	Ferguson & Baguley (1988)
Salmonella typhimurium, TA97a, TA98, TA100, TA102	Reverse mutation	–	–	10 µg/plate	Malachite green oxalate; purity, 70.8%	Fessard et al. (1999)
Salmonella typhimurium, TA1535, TA100, TA102, TA104, TA98, TA97	Reverse mutation	–	–	10 µg/plate	Malachite green chloride	Culp & NTP (2004)
Salmonella typhimurium, TA100, TA1535, TA1537	Reverse mutation	–	–	160 µg/plate	Malachite green oxalate; cytotoxicity occurred at 1.28 µg/plate without S9	Clemmensen et al. (1984)
Salmonella typhimurium, strain cys_{19}^-	Reverse mutation	+	NT	0.1 mM	Purity, NR	Luck et al. (1963)
Escherichia coli, strain Sd-4-73	Reverse mutation	–	NT	One small crystal/plate	Malachite green oxalate; purity, NR	Szybalski (1958); Combes & Haveland-Smith (1982)
Escherichia coli, strain cis_6^-	Reverse mutation	+	NT	10 mM	Purity, NR	Luck et al. (1963)

Table 4.3 (continued)

Test system (species, strain)	End-point	Results[b]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Leucomalachite green						
Salmonella typhimurium, TA97a, TA98, TA100, TA102	Reverse mutation	–	–	2000 µg/plate	Purity, NR	Fessard et al. (1999)

DAPI, 4,6-diamidino-2-phenylindole; HIC, highest ineffective concentration; LEC, lowest effective concentration; NA, not applicable; NR, not reported; NT, not tested; ppm, parts per million; RAPD, random amplification of polymorphic DNA; S9, 9000 × *g* supernatant.

[a] Except where noted, the form of the agent that was tested was not specified.

[b] +, positive; –, negative.

No in vivo studies on DNA damage were available for leucomalachite green.

Gene mutation

Malachite green was not mutagenic in experimental animals. When female Big Blue B6C3F$_1$ transgenic mice were treated with malachite green at concentrations of up to 450 ppm in the feed, malachite green did not induce hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) mutations in lymphocytes from the spleen or cause c*II* mutations in liver cells (Mittelstaedt et al., 2004). Malachite green did not cause gene mutations (as measured by the recessive spot test) in mice given doses of up to 40 mg/kg bw (Jensen, 1984). [The Working Group noted that mutagenicity was not evaluated in Big Blue Fischer 344 transgenic rats.]

Leucomalachite green was mutagenic in female Big Blue B6C3F$_1$ transgenic mice. The significant increase in mutant frequency in the livers of female Big Blue mice persisted when the mutant frequencies were corrected for mutant independence and were confirmed in further *cll* mutational spectrum analysis (Mittelstaedt et al., 2004). Analysis of *cII* mutations in livers from mice treated with leucomalachite green in the feed revealed an increase of G→T and A→T transversions (Mittelstaedt et al., 2004). [The Working Group noted that the increase in G→T and A→T transversions is a typical mutation spectrum of mutations produced by bulky arylamine carcinogens.]

Leucomalachite green was not mutagenic in transgenic Fischer 344 Big Blue transgenic rats. The initial signal of mutagenicity, an increase in *lacI* mutant frequency (by plaque-forming unit screening) in livers from female rats at one dose (543 ppm) at only 16 weeks (Culp et al., 2002), was not confirmed when corrected for clonality (Manjanatha et al., 2004). In addition, the *lacI* mutational spectrum in rats treated with leucomalachite green was not significantly different from that found in controls ($P = 0.09$), indicating that the increase might be due to the disproportionate expansion of spontaneous *lacI* mutations (Manjanatha et al., 2004). No increase in mutation frequency was seen upon the re-analysis of the *cII* mutational spectrum in liver samples taken from female rats treated with leucomalachite green at 543 ppm in the feed for 16 weeks (Manjanatha et al., 2004). Leucomalachite green did not increase *Hprt* mutation frequency in spleen lymphocytes in either Big Blue rats (Manjanatha et al., 2004) or Big Blue mice (Mittelstaedt et al., 2004).

Chromosomal aberration

Malachite green caused chromosomal aberration in Swiss mice. In male Swiss mice, malachite green administered by gavage significantly increased the frequency of chromosomal aberrations and sister-chromatid exchanges in the bone marrow and spermatocytes (Donya et al., 2012). The responses occurred in a dose- and time-dependent manner. A significant increase in the frequency of chromosomal aberrations was observed in the bone marrow of Swiss albino female mice treated with malachite green at a dose of 4 mg/kg bw by intraperitoneal injection for 30 days (Das et al., 2013). Intake of the selenium compound DMSE can significantly decrease the effects of chromosomal aberration caused by malachite green (Das et al., 2013).

No in vivo studies on chromosomal aberration or sister-chromatid exchange were available for leucomalachite green.

Micronucleus formation

Several studies have investigated the induction of micronucleus formation by malachite green or leucomalachite green in rodents, and the majority of the results were negative. Das et al. (2013) reported a significant increase in the frequency of micronucleus formation in the bone marrow of Swiss albino female mice after intraperitoneal injection of malachite green at a dose of 4 mg/kg bw. Moreover, pre-treatment with

DMSE significantly decreased the frequency of micronucleus formation. An increase in the frequency of micronucleus formation was seen at the intermediate dose, but not at the highest dose, in blood erythrocytes from Big Blue B6C3F$_1$ mice treated with malachite green in the feed (Mittelstaedt et al., 2004). Similarly, no micronucleus formation was observed in the bone marrow of NMRI:BOM mice treated with malachite green oxalate by gavage at a single dose of 37.5 mg/kg bw (Clemmensen et al., 1984). Micronucleus formation was not induced in erythrocytes from B6C3F$_1$ mice given feed containing malachite green at concentrations up to 1200 ppm for 28 days; or in the bone marrow of Fischer 344 rats after three intraperitoneal injections at doses ranging from 1.1 to 8.8 mg/kg bw (Culp & NTP, 2004). [The Working Group noted that a small but significant increase was seen at the intermediate dose of 4.375 mg/kg bw.]

Leucomalachite green did not induce micronucleus formation in peripheral blood erythrocytes from B6C3F$_1$ mice exposed via feed; or in the bone marrow of Fischer 344 rats treated by intraperitoneal injection (Culp & NTP, 2004). Moreover, no micronucleus formation was seen in the bone marrow of Big Blue rats (Manjanatha et al., 2004) or in blood erythrocytes of Big Blue B6C3F$_1$ mice (Mittelstaedt et al., 2004; Culp & NTP, 2004) exposed to leucomalachite green via feed.

Sister-chromatid exchange

The frequency of sister-chromatid exchange was significantly increased in a dose- and time-dependent manner in the bone marrow of male Swiss mice treated with malachite green by gavage (Donya et al., 2012).

No in vivo studies on sister-chromatid exchange were available for leucomalachite green.

(ii) Non-human mammalian cells in vitro

See Table 4.2.

Several studies investigated the genotoxic effects of exposure to malachite green or leucomalachite green in non-human mammalian cells in vitro. The end-points included DNA damage, gene mutation, chromosomal aberration, and inhibition of DNA synthesis.

DNA damage

Fessard et al. (1999) reported that malachite green induced DNA damage in the absence and presence of metabolic activation, as measured by the comet assay, in Chinese hamster ovary (CHO)-K1 cells. In Syrian hamster embryo (SHE) cells, malachite green caused a concentration-related increase in the frequency of DNA strand breaks, as measured by alkaline elution assay (Panandiker et al., 1994; Mahudawala et al., 1999). Bose et al. (2005) reported a concentration-dependent increase in the frequency of DNA damage, as measured by the comet assay, in SHE cells. DNA damage, as measured by the comet assay, was seen in both SHE and transformed SHE cells (Ashra & Rao, 2006). Moreover, Cheng & Li (2009) showed that malachite green could form a fluorescent complex by intercalation with native double-strand calf thymus DNA in a concentration-related manner.

Leucomalachite green did not cause DNA damage in CHO-K1 cells in the absence or presence of metabolic activation (Fessard et al., 1999).

Gene mutation

Malachite green did not increase the number of thioguanine-resistant mutants in the CHO/*Hprt* mutation assay (Fessard et al., 1999). Malachite green was cytotoxic and its mutagenic potential could be evaluated only at very low concentrations (0.001–0.05 µg/mL medium in the absence of metabolic activation, or 0.1 µg/mL in the presence of metabolic activation).

Leucomalachite green was much less cytotoxic than malachite green, but also lacked mutagenicity in the *Hprt* assay (Fessard et al., 1999). In the absence of metabolic activation, the

mutation frequency was above that of controls at one concentration (75 µg/mL). In the presence of metabolic activation, an increased mutation frequency was observed at 5 µg/mL in one experiment (out of two), but significant changes were not observed at higher concentrations.

Chromosomal aberration

Malachite green did not increase the frequency of chromosomal aberration in CHO cells (Au & Hsu, 1979). Cells transformed with malachite green were found to be aneuploid in nature, as determined by flow cytometry and chromosomal pattern, with approximately 52% of the transformed cells having aneuploid chromosome numbers. Chromosomal aberrations were reported in SHE cells transformed with malachite green (Mahudawala et al., 1999).

No in vitro studies on chromosomal aberration were available for leucomalachite green.

(iii) Non-mammalian experimental systems in vivo and in vitro

See Table 4.3.

The genotoxic effects of malachite green and leucomalachite green have been studied in various non-mammalian experimental systems. The end-points included DNA damage, chromosomal aberration, micronucleus formation, and gene mutation.

DNA binding and DNA damage

Early studies showed that malachite green was able to intercalate and/or bind with DNA (Hadwiger & Schwochau, 1971; Rosenkranz & Carr, 1971). Müller & Gautier (1975) reported that malachite green interacted with DNA with a preference for A:T-rich areas. Fox et al. (1992) confirmed that, at lower concentrations, patterns of malachite green bound to DNA centred around A:T-rich regions with a slight preference for homopolymeric A and T, whereas at higher concentrations, malachite green bound to almost all available DNA sites. Souza et al. (2020) reported a significant increase in the frequency of DNA damage (as measured by the comet assay) in the erythrocytes of *Hemichromis bimaculatus* fish exposed to malachite green at a concentration of 0.75 mg/L for 4 days. Majeed et al. (2014) studied the binding effect of malachite green to polymerase chain reaction (PCR)-amplified linear DNA by the DNA electrophoretic mobility shift assay, and the results showed that malachite green was capable of strongly binding double-stranded DNA and causing its degradation.

Matsui (1980) showed that malachite green did not cause DNA damage in the rec assay with *Bacillus subtilis* strains NIG 17 rec^+ and NIG 45 rec^-. However, Kanhere et al. (2014) reported that malachite green had genomic effects (DNA damage) in *Chlorella pyrenoidosa*, as measured by random amplification of polymorphic DNA analysis. Exposure of the fish kidney cell line CSK or fish gill cell line CSG to malachite green for 48 hours caused concentration-dependent DNA damage, as measured by the comet assay, with a significantly increased frequency of DNA fragmentation at concentrations > 0.1 µg/mL (Majeed et al., 2014).

No studies on DNA binding or damage in non-mammalian experimental systems were available for leucomalachite green.

Chromosomal aberration

Malachite green caused chromosomal aberrations in several test systems. Early studies found that malachite green caused chromosomal aberrations in trout eggs (Lieder, 1961), and chromosomal derangement in *Chironomid* larvae (Keyl & Werth, 1959) and fruit flies (*Drosophila melanogaster*) (Pfeiffer, 1961). Shanmugam et al. (2017) showed that malachite green induced chromosomal and nuclear aberrations in the root tips of *Allium cepa*. Moreover, malachite green induced chromosomal aberrations in *Chlorella pyrenoidosa*, as measured by staining with the fluorochrome 4′,6-diamidino-2-phenylindole (Kanhere et al., 2014).

No studies on chromosomal aberrations in non-mammalian experimental systems were available for leucomalachite green.

Micronucleus formation

Souza et al. (2020) reported no significant alteration in the frequency of micronucleus formation in erythrocytes in *H. bimaculatus* ornamental fish exposed to malachite green at concentrations of up to 0.75 mg/L for 4 days. Svobodová et al. (1997) showed no significant increase in the frequency of micronucleus formation in erythrocytes in common carp exposed to malachite green at concentrations of 0.5 mg/L in water for 6 days when compared with controls.

No studies on micronucleus formation in non-mammalian experimental systems were available for leucomalachite green.

Gene mutation

The mutagenicity of malachite green and leucomalachite green has been studied in yeast and bacteria. Nagai (1959) showed that malachite green was an effective inducer of respiration-deficient mutations in baker's yeast, with minimal induction at a concentration of 1 mg/L (to produce 3% mutants) and optimal induction at a concentration of 3 mg/L (to produce 90% mutants). Because malachite green is very toxic to bacteria, it was mostly tested at low doses. In *Salmonella typhimurium* strain TA98 in the presence of metabolic activation, malachite green was mutagenic at concentrations of 30 µg/plate (Clemmensen et al., 1984) and 75 µg/plate (Ayed et al., 2017). A positive result was also observed in *S. typhimurium* strain cys_{19}^- with malachite green at a concentration of 0.1 mM (Luck et al., 1963). However, malachite green gave negative results in most of the *S. typhimurium* test strains – TA97, TA97a, TA98, TA100, TA102, TA104, TA1535, and TA1537 – in the presence or absence of metabolic activation (Ferguson & Baguley, 1988; Fessard et al., 1999; Culp & NTP, 2004; Cheriaa et al., 2012; Ayed et al., 2017). In *Escherichia coli*, malachite green gave negative results in strain Sd-4-73 (Szybalski, 1958); but positive results in strain cis_6^- at 10 mM (Luck et al., 1963).

Leucomalachite green was much less toxic than malachite green in bacteria, and there was no evidence of it being mutagenic in *S. typhimurium* strains TA97a, TA98, TA100, and TA102 at concentrations up to 2000 µg/plate (Fessard et al., 1999).

4.2.3 Induces oxidative stress

(a) Humans

No data were available to the Working Group.

(b) Experimental systems

See Table 4.4.

Exposure to malachite green has been associated with GSH depletion, lipid peroxidation, and oxidative-related enzyme activities in experimental systems. Significant depletion of GSH and an increase in lipid peroxides were seen in the livers of mice treated with malachite green by gavage (Donya et al., 2012). Das et al. (2013) reported a significant increase in levels of lipid peroxidation and significant decreases in levels of GSH and antioxidative enzymes glutathione-*S*-transferase, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase in mice treated with malachite green by intraperitoneal injection. Such induction could be significantly reduced by pre- or co-treatment with DMSE. Similarly, depletion of GSH and decreases in SOD, CAT, and glutathione peroxidase activities were also seen in mice treated orally with malachite green (Kasem et al., 2016).

Studies on reactive free-radical formation, analysed by electron spin resonance using 5,5-dimethyl-1-pyrroline *N*-oxide as a spin-trapping agent, showed that malachite green induced a dose-related increase in the generation of free radicals in SHE cells (Panandiker et al., 1993, 1994; Mahudawala et al., 1999).

Table 4.4 Oxidative stress-related biomarkers of malachite green in experimental systems

End-point/ biomarker	Species, strain (sex)/cell line	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Lipid peroxides	Mouse, Swiss albino (M)	Liver	↑	272 mg/kg bw per day (for 7, 14, 21, and 28 days)	Gavage, 7, 14, 21, and 28 days, 0, 272, and 543 mg/kg bw per day	Purity, NR	Donya et al. (2012)
GSH level	Mouse, Swiss albino (M)	Liver	↓	272 mg/kg bw per day (for 14, 21, and 28 days)	Gavage, 7, 14, 21, and 28 days, 0, 272, and 543 mg/kg bw per day	Purity, NR	Donya et al. (2012)
Lipid peroxidation	Mouse, Swiss albino (F)	Liver	↑	4 mg/kg bw per day	Intraperitoneal injection, 30 days, 100 µg/mouse (25 g bw)	One dose only; the control group did not receive intraperitoneal injection of solvent; purity, NR	Das et al. (2013)
GSH level GST, SOD, CAT, GPx activity	Mouse, Swiss albino (F)	Liver	↓	4 mg/kg bw per day	Intraperitoneal injection, 30 days, 100 µg/mouse (25 g bw)	One dose only; the control group did not receive intraperitoneal injection of solvent; purity, NR	Das et al. (2013)
GSH level SOD, CAT and GPx activity	Mouse, strain NR (M)	Liver	↓	5 mg/kg bw per day	Orally, 14 and 28 days, 0, 2.5, 5 mg/kg bw per day	Analytical grade	Kasem et al. (2016)
Reactive free radical formation, ESR-DMPO	Syrian hamster embryo cells, SHE	Cells	+	2 µg/mL	Cell culture (after adding DMPO), 100 mM	DMPO adduct formation measured by ESR; purity, NR	Panandiker et al. (1993)
Reactive free radical formation, ESR-DMPO	Syrian hamster embryo cells, SHE	Cells	+	1 µg/mL	Cell culture (after adding DMPO), 100 mM	DMPO adduct formation measured by ESR; purity, NR	Panandiker et al. (1994); Mahudawala et al. (1999)
MDA content, lipid peroxidation	Syrian hamster embryo cells, SHE	Cells	↑	0.025 µg/mL	Cell culture, 24 h, 0, 0.025, 0.05, and 0.1 µg/mL	Purity, NR	Panandiker et al. (1992, 1994)
SOD	Syrian hamster embryo cells, SHE	Cells	↓	0.1 µg/mL	Cell culture, 24 h, 0, 0.025, 0.05, and 0.1 µg/mL	Purity, NR	Panandiker et al. (1992)
CAT	Syrian hamster embryo cells, SHE	Cells	↑	0.025 µg/mL	Cell culture, 24 h, 0, 0.025, 0.05, and 0.1 µg/mL	Purity, NR; in a concentration-related manner	Panandiker et al. (1992)

bw, body weight; CAT, catalase; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; ESR, electron spin resonance; F, female; GPx, glutathione peroxidase; GSH, glutathione; GST, glutathione *S*-transferase; h, hour; HID, highest ineffective dose; LED, lowest effective dose; M, male; MDA, malondialdehyde; NR, not reported; SHE, Syrian hamster embryo; SOD, superoxide dismutase.

[a] +, positive; ↑, increase; ↓, decrease.

Increased lipid peroxidation, as measured by malondialdehyde content, was observed in SHE cells (Panandiker et al., 1992, 1994). A concentration-related increase in CAT activity was seen in SHE cells exposed to malachite green (Panandiker et al., 1992). A decrease in SOD activity was also seen in SHE cells exposed to malachite green (Panandiker et al., 1992).

No studies were available on the effects of leucomalachite green on oxidative stress in experimental systems.

4.2.4 Modulates receptor-mediated effects

See Table 4.5.

Estrogenic and anti-estrogenic activities of malachite green were studied in an uterotrophic assay. In an estrogenic assay, ovariectomized C57BL/6J mice were treated with malachite green by oral gavage at a dose of 100 mg/kg bw per day or by subcutaneous injection at 300 mg/kg bw per day for 7 days (Ohta et al., 2012). No estrogenic effects were seen for malachite green. In an anti-estrogenic assay, ovariectomized mice were co-treated with malachite green and ethynyl estradiol at a dose of 0.6 µg/kg bw by oral or subcutaneous administration. Only a slight but significantly antagonistic effect on estrogenic activity was seen after oral co-treatment (Ohta et al., 2012). Malachite green significantly decreased expression of the growth hormone receptor GHR1 in seabream primary hepatocytes (Jiao & Cheng, 2010).

The effects of malachite green and leucomalachite green on the blood levels of triiodothyronine (T3), thyroxine (T4), and thyroid-stimulating hormone (TSH) were studied in male and female rats given feed containing malachite green at 1200 ppm or leucomalachite green at 1160 ppm (Culp et al., 1999). For malachite green, T3 levels were significantly higher in treated rats than in the controls on day 21, and T4 levels were significantly lower in females treated with malachite green on both days 4 and 21. There were no significant changes in T3 or T4 levels in males, or in TSH levels in males or females (Culp et al., 1999). In male rats treated with leucomalachite green at 1160 ppm, there was a significant decrease in T4 levels and a significant increase in TSH levels on days 4 and 21 compared with the respective control groups (Culp et al., 1999).

Doerge et al. (1998) reported that leucomalachite green inhibited TPO-catalysed tyrosine iodination (half-maximal inhibition, IC_{50} = 5 µM) and the formation of thyroxines in the presence of low-iodine human goitre thyroglobulin (IC_{50} = 15 µM). The ability of malachite green and leucomalachite green to inhibit TPO-catalysed iodination and coupling reactions demonstrates the potential disruption of thyroid hormone homeostasis.

4.2.5 Causes immortalization

See Table 4.6.

Mahudawala et al. (1999) showed that injection of malachite green-transformed SHE cells into nude mice resulted in the development of sarcoma with a latency period of 2–3 months. Moreover, when the tumour from the first generation was transplanted into second-generation mice, tumour growth was shown within 7–10 days.

Several studies of cell transformation showed that exposure of SHE cells to malachite green resulted in morphologically transformed colonies in a concentration-related manner (Panandiker et al., 1993, 1994; Mahudawala et al., 1999).

Malachite green-induced malignant transformation of SHE cells was associated with enhanced expression of altered Tp53, Bcl2, and decreased sensitivity to apoptosis (Rao et al., 2000, 2001). Transformation was also associated with the abrogation of G2/M checkpoint control by elevated phosphorylation of Chk1 (checkpoint kinase 1, Chek1), decreased phosphorylation of Chk2 (Chek2), and decreased levels of cyclin

Table 4.5 Modulation of receptor-mediated effects by malachite green[a] and leucomalachite green in experimental systems

End-point/ biomarker	Species, strain (sex)/cell line	Tissue	Results[b]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Malachite green							
Estrogen agonistic effects	Mouse, C57BL/6J (ovariectomized F)	Uterine	–	100 mg/kg bw per day	Gavage, 7 days, 100 mg/kg bw per day at 24-h intervals	Malachite green base	Ohta et al. (2012)
Estrogen antagonistic effects	Mouse, C57BL/6J (ovariectomized F)	Uterine	+	100 mg/kg bw per day	Gavage, 7 days, 100 mg/kg bw per day at 24-h intervals	Malachite green carbinol base; co-treated with ethinyl estradiol at 0.6 µg/kg by gavage	Ohta et al. (2012)
Estrogen agonistic effects	Mouse, C57BL/6J (ovariectomized F)	Uterine	–	300 mg/kg bw per day	Subcutaneous injection, 7 days, 300 mg/kg bw per day at 24-h intervals	Malachite green carbinol base	Ohta et al. (2012)
Estrogen antagonistic effects	Mouse, C57BL/6J (ovariectomized F)	Uterine	–	300 mg/kg bw per day	Subcutaneous injection, 7 days, 300 mg/kg bw per day at 24-h intervals	Malachite green carbinol base; co-treated with ethinyl estradiol at 0.6 µg/kg subcutaneously	Ohta et al. (2012)
GHR1	Seabream, primary hepatocytes	Liver	↓	0.1 nM	0.1, 1, 10, and 100 nM	Decrease not significant for GHR2 and no changes for IGF-I	Jiao & Cheng (2010)
T3	Rat, F344:N Nctr BR (M, F)	Blood	↑	1200 ppm	Gavage, 4 or 21 days, 1200 ppm	Malachite green chloride Increase at 21 days only in female rats	Culp et al. (1999)
T4	Rat, F344:N Nctr BR (M, F)	Blood	↓	1200 ppm	Gavage, 4 or 21 days, 1200 ppm	Decrease at 4 and 21 days only in female rats	Culp et al. (1999)
TSH	Rat, F344:N Nctr BR (M, F)	Blood	–	1200 ppm	Gavage, 4 or 21 days, 1200 ppm		Culp et al. (1999)
Leucomalachite green							
T3	Rat, F344:N Nctr BR (M)	Blood	–	1160 ppm	Gavage, 4 or 21 days, 1160 ppm		Culp et al. (1999)
T4	Rat, F344:N Nctr BR (M)	Blood	↓	1160 ppm	Gavage, 4 or 21 days, 1160 ppm	Decrease on days 4 and 21	Culp et al. (1999)
TSH	Rat, F344:N Nctr BR (M)	Blood	↑	1160 ppm	Gavage, 4 or 21 days, 1160 ppm	Increase on days 4 and 21	Culp et al. (1999)
MIT (3-iodotyrosine)	Porcine TPO	Acellular testing system	↓	5 µM	TPO-catalysed tyrosine iodination, NR, 0, 5, 15, and 30 µM	TPO activity	Doerge et al. (1998)

Table 4.5 (continued)

End-point/ biomarker	Species, strain (sex)/cell line	Tissue	Results[b]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
T3 and T4 residues	Porcine TPO	Acellular testing system	↓	15 µM	TPO-catalysed tyrosine iodination/coupling in thyroglobulin, NR, 0, 15, and 30 µM		Doerge et al. (1998)

bw, body weight; F, female; GHR, growth hormone receptor; h, hour; HID, highest ineffective dose; IGF, insulin-like growth factor; LED, lowest effective dose; M, male; MIT, monoiodotyrosine; NR, not recorded; ppm, parts per million; T3, triiodothyronine; T4, thyroxine; TPO, thyroid peroxidase; TSH, thyroid-stimulating hormone.

[a] Except where noted, the form of the agent that was tested was not specified.

[b] ↓, decrease; ↑, increase; +, positive; –, no effects.

Table 4.6 Cell transformation by malachite green in experimental systems

Species, strain (sex)/cell line	Tissue	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Mouse, nude (sex, NR)	Connective tissue	+	2 million MG-transformed SHE cells/mouse	2 million transformed cells were injected subcutaneously into dorsal side	Sarcomas produced with latency period of 2–3 mo	Mahudawala et al. (1999)
Mouse, nude (sex, NR)	Connective tissue	+	Part of tumour from first-generation mouse	Parts of tumours from the first-generation mice (amount, NR) were transplanted into nude mice	Tumour growth in 7–10 days	Mahudawala et al. (1999)
Syrian hamster embryo cells	Cell	+	0.025 µg/mL	Cell culture, 0.025, 0.05, 0.1, 1.0 µg/mL	Correlated with formation of reactive free radicals	Panandiker et al. (1993)
Syrian hamster embryo cells	Cell	+	1 µg/mL	Cell culture		Panandiker et al. (1994)
Syrian hamster embryo cells	Cell	+	0.025 µg/mL	Cell culture	Decrease in the number of foci at 0.05 µg/mL was due to cytotoxicity	Mahudawala et al. (1999)

HID, highest ineffective dose; LED, lowest effective dose; MG, malachite green; mo, month; NR, not reported.

[a] +, positive.

B1 (Ashra & Rao, 2006). Hyperphosphorylation of ERK2 (mitogen-activated protein kinase 1, Mapk1) and inhibition of JNK2 (mitogen-activated protein kinase 9, Mapk9) phosphorylation were observed during malachite green-induced transformation of SHE cells, which was associated with an increase in the number of cells in S phase (Bose et al., 2004). Furthermore, malachite green-induced transformation of SHE cells was associated with decreased expression of phosphoactive ERK and JNK and increased expression of p38 kinase (Bose et al., 2006).

No studies on immortalization were available for leucomalachite green.

4.2.6 Other key characteristics of carcinogens

Regarding whether malachite green alters cell proliferation, cell death, or nutrient supply, malachite green increased the number of liver eosinophilic foci in treated female rats (NTP, 2005). Malachite green acted as a potent liver tumour promoter (Fernandes et al., 1991; Rao & Fernandes, 1996; Gupta et al., 2003). Malachite green increased the expression of proliferating cell nuclear antigen (PCNA), upregulated cell cycle regulatory proteins, and stimulated DNA synthesis in hepatic preneoplastic lesions induced by *N*-nitrosodiethylamine in Wistar rats (Sundarrajan et al., 2000, 2001). Malachite green increased liver weight, the number of preneoplastic liver-cell foci, and the frequency of cell proliferation and apoptosis in preneoplastic liver-cell foci in rats after diethylnitrosamine initiation, effects that were ameliorated by apocynin and an antioxidant (Yoshida et al., 2017).

Increased cell proliferation in liver in F344/NS1c rats treated with a single dose of the initiator *N*-nitrosodiethylamine and with feed containing leucomalachite green was also reported (Kimura et al., 2016). In vitro, malachite green-transformed SHE cells showed enhanced DNA synthesis in the form of increased bromodeoxyuridine incorporation and expression of PCNA (Mahudawala et al., 2000).

Regarding whether malachite green or leucomalachite green is immunosuppressive, no data in mammalian species were available to the Working Group. In fish, reported increases or decreases in neutrophil or lymphocyte counts were transient and not consistent across the available studies, which were variable with respect to the species, exposure strategy, and concentrations tested (Bills & Hunn, 1976; Grizzle, 1977; Hlavek & Bulkley, 1980; Pickering & Pottinger, 1985; Svobodová et al., 1997; Saglam et al., 2003; Silveira-Coffigny et al., 2004; Yonar & Yonar, 2010; Witeska et al., 2013; Kwan et al., 2019).

4.3 Data relevant to comparisons across agents and end-points

The mechanistic characteristics common to carcinogens (the 10 key characteristics of carcinogens) can be investigated through biochemical and cell-based assays run by the United States Environmental Protection Agency (US EPA) and the United States National Institutes of Health Toxicity Forecaster/Toxicology in the 21st Century (ToxCast/Tox21) high-throughput screening programmes (Chiu et al., 2018; Guyton et al., 2018). Since 2017, the *IARC Monographs* have described the results of high-throughput screening assay to compare activity across agents and other in vitro and in vivo evidence relevant to the key characteristics. More information can be found in Section 4.4 of the monograph on gentian violet and leucogentian violet, in the present volume, including in Table 4.7, which summarizes findings for assay end-points mapped to key characteristics for the compounds evaluated. Details of the specific assays (and end-points) run for each chemical in this volume and the mapping to the key characteristics can be found in the Supplementary Material (Annex 1, Supplementary material for Section 4, web only;

available from: https://www.publications.iarc.fr/603).

4.3.1 Malachite green chloride

Malachite green chloride was considered active for 67 assay end-points (out of the 176 that were mapped to key characteristics) (US EPA, 2020b). Specifically, malachite green chloride was active in 1 of the 2 assay end-points mapped to "is genotoxic" and in all 5 of the 5 assay end-points mapped to "induces epigenetic alterations". Malachite green chloride was also considered active in 4 of the 10 assay end-points mapped to "induces oxidative stress", in 17 of the 50 assay end-points mapped to "modulates receptor-mediated effects", and 40 of the 63 assay end-points mapped to the "alters cell proliferation, cell death, or nutrient supply" key characteristic. Malachite green chloride was considered active in the H2AX (γ-H2AX) assay detecting DNA double-strand breaks in the CHO cell line CHO-K1, which is mapped to the "is genotoxic" key characteristic. Purity was not reported.

4.3.2 Malachite green oxalate

Malachite green oxalate (purity, > 50%) was considered active for 91 assay end-points (out of the 106 evaluated and mapped to key characteristics) (US EPA, 2020c). It was considered active in 1 of the 1 assay end-points mapped to "is electrophilic or can be metabolically activated to an electrophile", in 8 of the 9 assay end-points mapped to "is genotoxic", 3 of the 4 assay end-points mapped to "induces oxidative stress", 22 of the 32 assay end-points mapped to "modulates receptor-mediated effects", and 56 of the 58 assay end-points mapped to the "alters cell proliferation, cell death, or nutrient supply" key characteristic. Specifically, malachite green oxalate elicited TP53 activation measured through reporter assays in the human intestinal cell line HCT-116. Malachite green oxalate was considered active in the H2AX (γ-H2AX) assay, which detects protein phosphorylation, consistent with DNA double-strand breaks in the CHO cell line CHO-K1. Malachite green oxalate was also considered active in assays using DT40 chicken lymphoblastoid cell lines deficient for the DNA repair genes *REV3*, *KU70*, and *RAD54*. Malachite green oxalate was not considered active in the ATAD5-luc assay in HEK293T cells, which measures levels of ATAD5 protein that localize to the site of stalled replication forks resulting from DNA damage in replicating cells.

4.3.3 Leucomalachite green

Leucomalachite green (purity, > 90%) was considered active for 44 assay end-points (out of 236 assay end-points evaluated) (US EPA, 2020b): 2 of the 10 mapped to "is genotoxic", 4 of the 13 mapped to "induces oxidative stress", 1 out of 47 mapped to "induces chronic inflammation", and 24 of the 91 mapped to "alters cell proliferation, cell death, or nutrient supply". Leucomalachite green was considered active for 13 of the 69 assay end-points evaluating "modulates receptor-mediated effects". Relevant to DNA damage, leucomalachite green was considered active in the two assays using DT40 chicken lymphoblastoid cell lines deficient for the DNA repair genes *REV3* and *KU70/ RAD54*.

4.3.4 Summary

Malachite green chloride, malachite green oxalate, and leucomalachite green have been evaluated in ToxCast or Tox21 assays with end-points mapped to key characteristics of carcinogens. These compounds were active in a significant fraction of mapped end-points in which they have been tested (38% for malachite green chloride, 86% for malachite green oxalate, and 19% for leucomalachite green).

Specifically, malachite green oxalate was considered active in most of the "is genotoxic"

assay end-points. Malachite green oxalate and malachite green chloride were considered active in all the "induces epigenetic alterations" assay end-points. In addition, these compounds were considered active for a variety of the assay end-points mapped to the following key characteristics: induces oxidative stress, modulates receptor-mediated effects, and alters cell proliferation, cell death, or nutrient supply. Relevant to findings in other sections, malachite green oxalate, and leucomalachite green were considered active in an assay measuring thyroid receptor antagonism in GH3, a rat pituitary gland cell line, and these compounds were considered to give negative results in an assay measuring thyroid hormone receptor-agonist activity in the same cell line. Malachite green chloride, and leucomalachite green were considered to give negative results in an assay measuring thyroid hormone receptor-mediated transcription in HepG2 cells.

5. Summary of Data Reported

5.1 Exposure characterization

Malachite green is a cationic triphenylmethane dye. The reduced form of malachite green is leucomalachite green, which can be formed by chemical or enzymatic reduction of malachite green. Malachite green is widely used for dyeing a wide variety of materials, including textiles, paper, acrylic products, and hair dyes. It is used as a biological stain, an analytical reagent, and as a pH indicator. Besides its use as a dye, malachite green is also an aquarium disinfectant and as an antiparasitic, antifungal, and antibacterial agent in aquaculture. Leucomalachite green is used as a dye precursor to malachite green, as a reagent in several analytical applications, and as a radiochromic indicator in dosimeters to detect radiation exposure. As malachite green may be used to control fish diseases, residues of its major metabolite, leucomalachite green, might be found in treated fish or shellfish and have a longer residence time than the parent compound.

Malachite green may be released into the environment from waste discharge by textile mills and after other industrial production or processing, and persists in soil and aquatic species primarily as leucomalachite green.

Overall, data on exposure to malachite green and leucomalachite green are sparse. The potential for occupational exposure to malachite green and leucomalachite green exists through dermal contact and inhalation at workplaces where these compounds are produced or applied; however, few data on populations that have been exposed occupationally or occupational exposure levels were identified.

In the general population, exposure can occur through contact with textile, paper, inks, and hair dye containing malachite green; through the occasional treatment of diseased ornamental and farmed fish and shellfish with malachite green; and the consumption of fish or shellfish containing residues of malachite green and leucomalachite green. One study indicated that the use of hair dyes and the consumption of drinking-water may be important routes of exposure to malachite green.

Malachite green is not authorized for use as a veterinary drug, for cosmetic applications, or for food packaging in many countries, and there is zero tolerance for residues of malachite green and its marker, leucomalachite green, in food for human consumption.

5.2 Cancer in humans

No data were available to the Working Group.

5.3 Cancer in experimental animals

5.3.1 Malachite green

Exposure to malachite green caused an increase in the incidence of an appropriate combination of benign and malignant neoplasms in one sex (female) of a single species (rat) in a study that complied with Good Laboratory Practice (GLP).

In female F344/N Nctr Br rats exposed to malachite green chloride in the feed, there was a significant positive trend and significant increase in the incidence of follicular cell adenoma or carcinoma (combined) of the thyroid gland in a study that complied with GLP.

5.3.2 Leucomalachite green

Exposure to leucomalachite green caused an increase in the incidence of an appropriate combination of benign and malignant neoplasms in one sex (female) of one species (mouse) in a study that complied with GLP, and in males and females of another species (rat) in a study that complied with GLP.

There was a significant positive trend and significant increase in the incidence of hepatocellular adenoma or carcinoma (combined) in female B6C3F_1/Nctr Br mice exposed to leucomalachite green in the feed in a study that complied with GLP. In another species, leucomalachite green in the feed increased the incidence of follicular cell adenoma or carcinoma (combined) of the thyroid gland in male F344/N Nctr Br rats, which was treatment-related, and significantly increased the incidence of adenoma or carcinoma (combined) of the mammary gland in female F344/N Nctr Br rats in a study that complied with GLP.

5.4 Mechanistic evidence

No direct data on absorption, distribution, metabolism, or excretion of malachite green in humans were available, but methaemoglobinaemia in a poisoning case provided indirect evidence of absorption and distribution. In orally dosed rats, excretion was primarily via the faeces. Various desmethyl malachite green derivatives and malachite green *N*-oxide were detected in liver extracts from Fischer 344 rats, but not from B6C3F_1 mice given feed containing malachite green or leuchomalachite green for 28 days. The metabolite leucomalachite green has been detected in the liver of rats exposed via the diet, in various rat tissues after intravenous injection, and in cultures of human and other mammalian intestinal microflora exposed to malachite green.

For malachite green, no mechanistic data from humans or human primary cells were available. Regarding the key characteristics of carcinogens, malachite green formed DNA adducts in the liver in a study of dietary exposure in male Fischer 344 rats and in female B6C3F_1 mice, but the adducts were not characterized. In one study in Swiss male mice treated by gavage, malachite green induced various clastogenic effects: hepatic DNA fragmentation, increased frequency of chromosomal aberrations, micronucleus formation, and sister-chromatid exchanges in bone marrow, and chromosomal aberrations in spermatocytes. In one study in female Swiss mice exposed intraperitoneally, malachite green induced hepatic DNA-strand breaks, as well as chromosomal aberrations and micronucleus formation in the bone marrow. Hepatic DNA damage was reported in one additional study of oral exposure in an unspecified mouse strain. On the other hand, malachite green did not induce micronucleus formation in other mouse strains and in rats, in experiments examining the blood erythrocytes of male and female B6C3F_1 mice or Big Blue B6C3F_1 transgenic female mice after dietary exposure, the bone marrow of an

NMRI:BOM mouse exposed once by gavage, or the bone marrow of Fischer 344 male rats after three intraperitoneal exposures. Malachite green was not mutagenic in the mouse spot test in C57B1/6J mice or the gene mutation assay in Big Blue B6C3F$_1$ transgenic mice. The differences in study outcome across strains could not be explained by the different routes or doses of exposure, or study quality, including the purity of the agent tested.

Malachite green was considered active in various high-throughput in vitro assays indicative of DNA damage, including TP53 activation and γH2AX, and in an assay of DNA damage in DT40 chicken lymphoblastoid cells deficient in DNA-repair genes. In other studies in cultured hamster cells, malachite green induced DNA damage but the results for chromosomal aberrations were mixed in the two available studies. DNA damage and chromosomal aberrations were reported in fish and plants. Malachite green gave largely negative results for mutagenicity across various *Salmonella typhimurium* and *Escherichia coli* strains.

Malachite green increased lipid peroxidation, and decreased glutathione levels and antioxidant enzyme activity in mice. Oxidative stress was also induced in cultured rodent and fish cells. No direct measurements of oxidative damage to DNA by malachite green were available, although one study showed that malachite green-induced DNA damage was significantly blocked by selenium or antioxidant enzymes. Malachite green increased cell proliferation and DNA synthesis, and increased the number of rat liver preneoplastic foci induced by *N*-nitrosodiethylamine. It induced malignant transformation of Syrian hamster embryo (SHE) cells.

Overall, a minority view among the Working Group held that the mechanistic evidence taken together is consistent and coherent based on findings supportive of DNA damage, clastogenicity, and oxidative stress. Malachite green induced DNA adducts in male rats and female mice in one study; DNA damage, chromosomal aberrations, and sister-chromatid exchanges in orally exposed male Swiss mice in one study; DNA damage, chromosomal aberrations, and micronucleus formation in intraperitoneally exposed female Swiss mice in one study; and DNA damage in hamster cells in several studies in vitro. The majority view, while finding that the evidence is suggestive of clastogenicity, considered that the relevant studies were few in number, narrow in range, and that the results were inconsistent. DNA damage was seen in two rodent species and in vitro; however, findings were inconsistent for micronucleus formation, for which the data were mostly negative in rodents and in vitro tests.

For leucomalachite green, the mechanistic evidence is suggestive of a carcinogenic effect. Regarding the key characteristics of carcinogens, leucomalachite green forms DNA adducts in the livers of male Fischer 344 rats, but not female B6C3F$_1$ mice, exposed via the diet. The DNA adducts have not been characterized. Leucomalachite green was considered active in the DT40 chicken lymphoblastoid high-throughput assay that is an indicator of DNA damage. It was mutagenic in the liver of Big Blue B6C3F$_1$ transgenic mice, inducing transversion mutations as confirmed via analysis of the mutation spectrum. It was not mutagenic in Big Blue rats. Leucomalachite green did not induce micronucleus formation in these two species. Data from the few available in vitro and non-mammalian tests were negative.

Significant changes in blood thyroid hormone levels were observed with malachite green in female rats, and leucomalachite green in male rats.

For other key characteristics of carcinogens, there is a paucity of available data.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *inadequate evidence* in humans regarding the carcinogenicity of malachite green.

There is *inadequate evidence* in humans regarding the carcinogenicity of leucomalachite green.

6.2 Cancer in experimental animals

There is *limited evidence* in experimental animals for the carcinogenicity of malachite green.

There is *sufficient evidence* in experimental animals for the carcinogenicity of leucomalachite green.

6.3 Mechanistic evidence

For malachite green, there is *limited mechanistic evidence*.

For leucomalachite green, there is *limited mechanistic evidence*.

6.4 Overall evaluation

Malachite green is *not classifiable as to its carcinogenicity to humans (Group 3)*.

Leucomalachite green is *possibly carcinogenic to humans (Group 2B)*.

6.5 Rationale

Malachite green was evaluated as *Group 3* because the evidence for cancer in experimental animals is *limited*, the mechanistic evidence is *limited*, and the evidence regarding cancer in humans is *inadequate*. The evidence for cancer in experimental animals is *limited* because there was an increase in the incidence of an appropriate combination of benign and malignant neoplasms, but only in one sex of a single species of animals in one study that complies with GLP. The mechanistic evidence is *limited* because findings in experimental systems are suggestive of clastogenicity, but the studies were few in number and narrow in range, and there were unresolved inconsistencies across different experimental studies. The evidence regarding cancer in humans is *inadequate* because no studies were available.

The *Group 2B* evaluation for leucomalachite green is based on *sufficient evidence* for cancer in experimental animals. The evidence regarding cancer in humans is *inadequate* as no studies were available. The mechanistic evidence is *limited* for leucomalachite green because findings in experimental systems are suggestive of mutagenicity, but the studies are few in number and narrow in range. The *sufficient evidence* for cancer in experimental animals is based on an increase in the incidence of an appropriate combination of benign and malignant neoplasms in both sexes of one species in one study that complies with GLP, and in one sex of another species in another study that complies with GLP.

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CI DIRECT BLUE 218

1. Exposure Characterization

1.1 Identification of the agent

Colour Index (CI) Direct Blue 218 is a bis copper-chelated dimethoxybenzidine-based azo dye. Azo dyes are diazotized amines coupled to an amine or phenol, with one or more azo bonds (R–N=N–R′). The azo group constitutes the chromophore of the dye, i.e. the chemical group primarily responsible for colour (Aspland, 1991). Azo dyes are the most structurally diverse class of organic dyes, with over 3000 azo dyes having been available in the past and 2000 dyes currently available (Chung, 2016).

The essential precursors of azo dyes are aromatic amines (Chung, 2016). CI Direct Blue 218 is based on benzidine or its congeners as precursors (Morgan et al., 1994). Dyes are metallized, in this case with copper, to improve the stability of the azo groups and increase light and wash fastness (Aspland, 1991; Morgan et al., 1994).

CI Direct Blue 218 is a direct dye, also called a substantive dye. Direct dyes have a natural affinity for cellulose without the need for a mordant (Waring & Hallas, 1990; Aspland, 1991). Direct dyes, which mostly belong to the azo class, are superior to others in terms of cost, light fastness, ease of application, short durations of dye cycles, low cost of auxiliaries, remarkably lower use of water, and much lower levels of effluent salt (Textile Property, 2021).

The CI is used to number dyes with respect to their application class and shade using a sequential numbering system (Morgan et al., 1994). The CI constitution number is 24 401 for the dye with the CI generic name CI Direct Blue 218.

1.1.1 Nomenclature

Chem. Abstr. Serv. Reg. No.: 28407-37-6

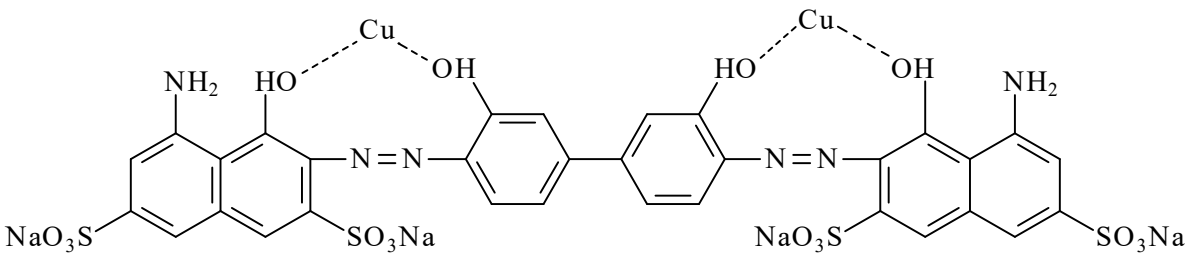
Chem. Abstr. Serv. name: cuprate(4-), [μ-[[3,3′-[[3,3′-di(hydroxy-κO)[1,1′-biphenyl]-4,4′-diyl]bis(2,1-diazenediyl-κN1)]bis[5-amino-4-(hydroxy-κO)-2,7-naphthalenedisulfonato]](8-)]]di-, sodium (1:4)

EC No.: 249-008-8 (ECHA, 2022)

IUPAC systematic name: tetrasodium; 5-amino-3-[[4-[4-[(8-amino-1-hydroxy-3,6-disulfonatonaphthalen-2-yl)diazenyl]-3-hydroxyphenyl]-2-hydroxyphenyl]diazenyl]-4-hydroxynaphthalene-2,7-disulfonate; copper (NCBI, 2020)

Synonyms: DIRECT BLUE 218, 28407-37-6; CI 24 401; Fastusol Blue 9GLP; Solantine Blue 10GL; Pontamine Bond Blue B; Amanil Supra Blue 9GL; CI Direct Blue 218; Pontamine Fast Blue 7GLN; UNII-RR3V5FL20N; RR3V5FL20N; CCRIS 6142; HSDB 4223; NCI C60 877; EINECS 249-008-8;

Fig. 1.1 Chemical structure of CI Direct Blue 218



INTRALITE Blue 8GLL; DIRECTBLUE218; (3,3′-((3,3′-Dihydroxy-1,1′-biphenyl-4,4′-diyl)bis(azo)bis(5-amino-2,7-naphthalenedisulfonato-(O4,O3)))dicopper, tetrasodium salt; 2,7-Naphthalenedisulfonic acid, 3,3′-((3,3′-dihydroxy(1,1′-biphenyl)-4,4′-diyl)bis(azo)bis(5-amino-4-hydroxy-, sodium salt, copper complex; Copper, [mu-[tetrahydrogen-3,3′-[(3,3′-dihydroxy-4,4′-biphenylylene)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonato](4-)]di-, tetrasodium salt; Cuprate(4-), [mu-[[3,3′-[(3,3′-dihydroxy[1,1′-biphenyl]-4,4′-diyl)bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonato]](8-)]]di-, tetrasodium; 2,7-Naphthalenedisulfonic acid, 3,3′-[(3,3′-dihydroxy[1,1′-biphenyl]-4,4′-diyl) bis(azo)]bis[5-amino-4-hydroxy-, sodium salt, copper complex; 1-Naphthol-3,6-disulfonic acid, 2,2′-(3,3-dihydroxy-4,4′-dipiphenylylenebisazo)bis[8-amino-, dicopper deriv., tetrasodium salt (CAMEO, 2020; NCBI, 2020).

1.1.2 Structural and molecular formulae, and relative molecular mass

The chemical structure of CI Direct Blue 218 is provided in Fig 1.1.

Molecular formula: $C_{32}H_{20}Cu_2N_6Na_4O_{16}S_4$ (PubChem CID: 34237 or 137241407)

Relative molecular mass: 1091.9 (powder form)

A solution form of this dye is indicated as PubChem CID: 24832073, with the molecular formula $C_{32}H_{16}Cu_2N_6Na_4O_{16}S_4$ and a relative molecular mass of 1087.8.

[The Working Group noted that the chemical and physical properties presented below are for the powder form.]

1.1.3 Chemical and physical properties of the pure substance

Description: deep purple to dark blue amorphous powder (NTP, 1992; ECHA, 2020a)

Boiling point: 1560 °C (estimated, based on EPI Suite MPVPBP V1.43) (ECHA, 2020a)

Melting point: 350 °C (estimated, based on EPI Suite MPVPBP V1.43) (ECHA, 2020a)

Density: 2.8 ± 0.1 g/cm^3 at 20 °C (ECHA, 2020a)

Solubility: 1.00–5.00 × 10^{-4} mg/L at 17 °C in water (ECHA, 2020a)

Vapour pressure: 1.06 × 10^{-39} Pa at 25 °C (ECHA, 2020a)

Flash point: flash-point data for this chemical are not available; however, it is probably combustible (NTP, 1992)

Stability and reactivity: CI Direct Blue 218 is a diazo compound. Azo, diazo, and azido compounds can detonate. Their nitro groups facilitate rapid decomposition. This particularly applies to organic azides that have been sensitized by the addition of metal salts or strong acids. Toxic gases are formed by mixing materials of this class with acids, aldehydes, amides, carbamates, cyanides, inorganic fluorides, halogenated organics, isocyanates, ketones, metals, nitrides, peroxides, phenols, epoxides, acyl halides, and strong oxidizing or reducing agents. Flammable gases are formed by mixing materials in this group with alkali metals. Explosive combination can occur with strong oxidizing agents, metal salts, peroxides, and sulfides (CAMEO, 2020).

Octanol/water partition coefficient (P): log K_{ow}, −0.77 (ECHA, 2020a).

1.1.4 Impurities

The purity of CI Direct Blue 218 was reported to be approximately 60% in a study by the National Toxicology Program (NTP) (NTP, 1994). Chemical characterization of two lots characteristic of the product used by industry indicated more than 12 impurities, of which the majority appeared to contain the reducible azo bond (Morgan et al., 1994; NTP, 1994). No attempt was made to identify the chromatographic peaks; however, reduction titration of azo groups indicated that the two lots had purities of 90% and 83%. The concentrations of benzidine and 3,3′-dimethoxybenzidine were determined. Benzidine could not be detected in either lot at levels greater than 1 ppm [1 µg/mL]. 3,3′-Dimethoxybenzidine was found at levels less than or equal to 7 ppm [7 µg/mL], but the level was less than 1 ppm in the lot used for a 2-year bioassay (Morgan et al., 1994; NTP, 1994). The study by the NTP also noted that other impurities, in addition to those containing azo groups, probably included inorganic copper salts.

[The Working Group noted that the only data on impurities were derived from the above-mentioned assays reported by the NTP in 1994. However, several manufacturers listing CI Direct Blue products online in 2021 were claiming a purity of 96–99%.]

1.2 Production and use

1.2.1 Production process

CI Direct Blue 218 is produced by coupling one mole of *ortho*-dianisidine (3,3′-dimethoxybenzidine) to two moles of 4-amino-5-hydroxy-2,7-naphthalene disulfonic acid under alkaline pH conditions (resulting in CI Direct Blue 15), followed by the addition of a copper salt and the elimination of methyl groups from the methoxides to form a copper complex (Kirk-Othmer, 1978). Due to the copper chelation process, the substance does not contain methoxy groups that are characteristic of the *ortho*-dianisidine (3,3′-dimethoxybenzidine) moiety, which is a major component of the dye (NIOSH, 1980).

1.2.2 Production volume

CI Direct Blue 218 is listed by the Organisation for Economic Co-operation and Development (for the year 2007) as a High Production Volume chemical (OECD, 2009). The production volumes in the USA were 4.03×10^5 kg in 1977, 3.54×10^5 kg in 1979, and 3.33×10^5 kg in 1985 (NCBI, 2003). In 1994, there were four production plants registered in the USA (NCBI, 2003).

Production or import volumes in the USA were reported to be between 10 000 and 500 000 pounds [between 4.5 and 230 tonnes] in 1986, 1990, 1994, 1998, and 2002, and < 500 000 pounds [< 230 tonnes] in 2006 (US EPA, 2003, 2007; IARC, 2010). The national aggregated production volumes in the USA

according to Chemical Data Reporting records were < 1 000 000 pounds [< 450 tonnes] per year from 2012 to 2015 (US EPA, 2020). In 2020, CI Direct Blue 218 was available from two suppliers in the USA and 12 suppliers from China (Chemical Register, 2020). Based on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) registration data reported in 2018, between 1 and 10 tonnes of CI Direct Blue 218 were manufactured or imported in the European Economic Area (ECHA, 2020a).

[The Working Group noted that no specific information could be found on production volumes of CI Direct Blue 218 in China or elsewhere outside of the USA.]

1.2.3 Use

CI Direct Blue 218 is used as a dye for cellulose, acetate, nylon, silk, wool, tissue, fine papers, and textile goods with a urea-formaldehyde finish (NCBI, 2003; ECHA, 2020a). As noted in the introduction to this section, direct dyes have a natural affinity for cellulose without the need for a mordant (Waring & Hallas, 1990; Aspland, 1991). Azo dyes are synthetic compounds with vivid colours, of which > 7 × 10^5 tons [> 6.4 × 10^5 tonnes] are produced annually worldwide, accounting for > 50% of all dyestuffs produced worldwide (Chung, 2016). Boeniger (1980) reported the total use of benzidine-based dyes as follows: 40% to colour paper, 25% to colour textiles, 15% to colour leather, and 20% in diverse applications in the petroleum, rubber, plastics, wood, soap, fur, and hair dye industries (Boeniger, 1980). [The Working Group noted that these data were not specific to CI Direct Blue 218, for which no data were available.]

1.3 Methods of detection and quantification

1.3.1 Air

No specific methods are available for the measurement of CI Direct Blue 218 in air. National Institute for Occupational Safety and Health (NIOSH) method No. 5013 is available for benzidine-based dyes (NIOSH, 1994). This method also can be used for *ortho*-dianisidine-based (3,3′-dimethoxybenzidine-based) dyes. Sampling of airborne dyes is performed with a 5 µm polytetrafluoroethylene membrane filter with a flow rate of 1–3 L/minute. After ultrasonic treatment of the filter, sodium hydrosulfite is added for reductive cleavage of the dye; the isolated benzidine is finally analysed by high-performance liquid chromatography-ultraviolet (HPLC-UV). The estimated limit of detection (LOD) of the method is 6–20 µg/m^3.

1.3.2 Other environmental media

Few specific methods are available for the measurement of CI Direct Blue 218 and dimethoxybenzidine-based dyes in environmental media. A method for the chemical characterization of CI Direct Blue 218 was used that applied thin-layer chromatography and HPLC with UV/visible light detection at 254 and 658 nm (NTP, 1994). CI Direct Blue 218 was also measured in feed following extraction with methanol or methanol with tetrabutylammonium hydroxide by spectrophotometric measurement of the 622 nm absorbance maximum. Different analytical methods are used for the analysis of 3,3′-dimethoxybenzidine and dimethoxybenzidine-based dyes in a variety of matrices (water, paint, textiles, food, and toys). In general, methods involve reductive cleavage of the dye to the free amine and analysis of the resultant amines. [The Working Group noted that, in the case of CI Direct Blue, it is unlikely that 3,3′-dimethoxybenzidine is

formed after cleavage, since the methoxy groups are no longer present. Instead 3,3′-dihydroxybenzidine is more likely to be formed. The analytical methods described below are also relevant for these products because they detect the amine groups.]

While gas chromatography (GC) analysis invariably requires derivatization of the amine before the analysis, analysis by liquid chromatography (LC) in combination with mass spectrometry (MS) does not. Also, the use of modern liquid chromatography with tandem mass spectrometry (LC-MS/MS) methods permits the analysis of complex mixtures. A method based on the analysis of amine with HPLC-UV (after reductive cleavage) has been developed for 3,3′-dimethoxybenzidine-based dyes in toys, with an estimated LOD of 0.2 µg/g (Garrigós et al., 2002). For wastewater, a method has been developed by dissolving the dyes in methanol, dichloromethane, or ethylacetate before analysis with GC-MS (Doherty, 2005). For textiles, a method that involves refluxing with chlorobenzene followed by three consecutive extractions with citrate buffer, hydrosulfite, and *tert*-methyl ether was developed for use with LC-MS/MS analysis (Sutthivaiyakit et al., 2005). This method forms the basis for the later-developed International Organization for Standardization (ISO) method 14362-1:2017 (ISO, 2017). In the ISO method, several chromatographic techniques and detection methods are described: HPLC with diode-array detection, HPLC-MS, GC-MS, and GC-flame ionization detection. The estimated LOD of the method is 5 µg/g.

1.3.3 Biological specimens

No biomonitoring methods for the assessment of exposure to CI Direct Blue 218 or its possible metabolites were identified in the literature.

1.4 Occurrence and exposure

1.4.1 Environmental occurrence

A study of the Yamaska River in Quebec, Canada, conducted between 1985 and 1987, investigating the occurrence of 15 dyes in river water, sediments, and solids, as well as fish downstream of textile mills, did not detect CI Direct Blue 218 (Maguire, 1992). No other environmental studies on CI Direct Blue 218 were identified. Very little is known about the environmental occurrence, persistence, and fate of individual dyes because of difficulties in determining different chemical classes of dyes at trace levels in environmental samples (Maguire, 1992). Benzidine and its congeners are not known to occur naturally in the environment. Although few actual measurements of the release of benzidine-based compounds into the environment have been reported, it is thought that manufacturing and processing plants for dyes and pigments derived from benzidine and its congeners are the major sources of release. Three major sources of environmental release of dyes and pigments derived from benzidine and its congeners have been identified: process wastewaters; atmospheric release; and disposal of dyed articles (US EPA, 1980).

About 10% of the azo dyes used in textile dyeing processes are released into the environment (Chung, 2016; dos Santos, 2018). In the 1980s, it was estimated that, worldwide, 280 000 tons [units assumed to be imperial tons; 284 494 metric tonnes] of textile dyes were annually discharged into industrial effluents (Chung, 2016). Since the azo dyes represent about 70% by weight of the dyestuffs used, it follows that they are the most common group of synthetic colourants released into the environment (Chung, 2016).

Generally, ionic azo dyes released into surface waters or wastewater are expected to bind primarily to suspended organic matter,

due to electrostatic interactions, and ultimately sequester to sediments or wastewater sludge. However, a proportion of ionic dyes are likely to remain dissolved in the water column (dos Santos, 2018). Because of their stability and microbial resistance, azo dyes are not readily removed from wastewater by conventional treatment methods (Chung, 2016). Contaminated sewage sludge is often dumped in landfill waste sites, resulting in soil and groundwater contamination (Tkaczyk et al., 2020). Furthermore, sludge from wastewater treatment plants or industrial wastewater may be applied to agricultural fields, which may result in significant concentrations of dyes in agricultural soils (dos Santos, 2018; Tkaczyk et al., 2020). Complexed metal dyes have half-lives of 2–13 years and in the aquatic environment the heavy metal cations can be assimilated by fish gills, which can lead to accumulation in certain tissues (Lellis et al., 2019). As a consequence of both the direct and indirect sources described above, synthetic organic dyes pass through different trophic levels of the food web, from (water) plants and algae through consumers of the first order (e.g. crustaceans) and through secondary consumers (e.g. fish) to humans. Since this may cause biomagnification of dye components in the food chain, these compounds are considered to be persistent bioaccumulative toxic substances (Tkaczyk et al., 2020).

1.4.2 Occupational exposure

From a survey conducted between 1981 and 1983, NIOSH estimated that a total of 12 290 workers might come into contact with CI Direct Blue 218 in the textile and paper industries (NIOSH, 2017). Industrial exposure to dyes may occur through inhalation of dust or mist, accidental ingestion, or direct contact with the skin (NIOSH, 1980). Potential exposure to CI Direct Blue 218 occurs during the manufacturing process (synthesis, processing, packaging, transportation, or maintenance and clean-up), from the application of the dye on products, and from further processing of dyed products that results in particles being formed (NIOSH, 1983).

1.4.3 Exposure in the general population

No data on exposure to CI Direct Blue 218 in the general population have been identified; however, most environmental exposure to 3,3′-dimethoxybenzidine and 3,3′-dimethoxybenzidine-based dyes has been described as resulting from contact with contaminated air, water, or soil. In addition, the general population may also be exposed to 3,3′-dimethoxybenzidine-based dyes via contact with paper or fabric products containing these dyes, or through consumer use of the dyes (NTP, 2016). However, the benzidine-based dyes in finished products are not considered to migrate from the product as a result of washing, perspiration, or contact with saliva (US EPA, 1980).

1.5 Regulations and guidelines

CI Direct Blue 218 is very toxic to aquatic life with long-lasting effects (H410) and causes serious eye irritation (H319) (ECHA, 2020a). There are additional regulations and guidelines for overarching groups of dyes to which CI Direct Blue 218 may belong. For dimethoxybenzidine-based azo dyes, these are Cosmetics Directive 1223/2009/EC (European Commission, 2009) and the Opinion of the Scientific Committee on Cosmetic and Non-Food Products intended for Consumers (SCCNFP) (SCCNFP, 2002). For dyes that are metabolized to aromatic amines, the following are available: REACH Annex XVII (ECHA, 2020b); Plastic Food Contact Materials Directive 10/2011/EC (European Commission, 2011); Toys Safety Directive: EN-71 standards (European Committee for Standardization, 2014); and guidelines from the United States Occupational Safety and Health Administration (OSHA) and NIOSH (NIOSH, 1980).

2. Cancer in Humans

No data were available to the Working Group.

3. Cancer in Experimental Animals

See Table 3.1.

3.1 Mouse

Oral administration (feed)

In a study of chronic toxicity and carcinogenicity that complied with Good Laboratory Practice (GLP) and that was conducted by the NTP (1994), groups of 50–51 male and 50 female B6C3F$_1$ mice (age, 7 weeks) were given feed containing CI Direct Blue 218 (a desalted commercial dye of 60% copper complex of 3,3′-[(3,3′-dihydroxy[1,1′-biphenyl]-4,4′-diyl) bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid] tetrasodium salt; 11% water, 0.7% sodium chloride; reduction titration of azo groups indicated a purity of 83%, and the authors stated that the titration estimate of purity was "probably enhanced by the presence of reducible low molecular weight organic impurities containing the azo group as well as inorganic copper salts"; 3,3′-dimethoxybenzidine was < 1 ppm, and benzidine was ≤ 1 ppm) at a concentration of 0, 1000, 3000, or 10 000 ppm (approximately equal to average daily doses of 0, 120, 360, and 1520 mg/kg body weight (bw) per day for males and 0, 140, 470, and 2050 mg/kg bw per day for females, respectively), for the control group and groups at the lowest, intermediate, and highest dose, respectively, for 104 weeks. In addition, 9 males for the control group and 10 males and 10 females for all the other groups were given feed containing CI Direct Blue 218 at a concentration of 0 (controls), 1000, 3000, or 10 000 ppm, respectively, for interim evaluation at 15 months. Survival of exposed male and female mice was similar to that of the controls. At study termination (104 weeks), survival was 44/50, 46/50, 42/50, and 45/50 in males, and 37/49, 40/50, 46/49, and 38/49 in females, for the control group and groups at the lowest, intermediate, and highest dose, respectively. The final mean body weight of mice at the highest dose was 10 000 ppm was 19% lower than that of the control mice for males and 27% lower than that of the control mice for females. Complete necropsies and full histopathological examinations were performed.

In male mice, there was a significant positive trend in the incidence of hepatocellular adenoma, hepatocellular carcinoma, and hepatocellular adenoma or carcinoma (combined) ($P < 0.001$, logistic regression trend test), with a significant increase in the incidence of hepatocellular adenoma, hepatocellular carcinoma, and hepatocellular adenoma or carcinoma (combined) at the highest dose ($P < 0.001$; $P = 0.019$; $P < 0.001$, respectively, logistic regression test). Renal tubule adenomas were observed in two males at the lowest dose, one male at the intermediate dose, and one male at the highest dose. In addition, one renal tubule carcinoma was observed in another male at the lowest dose. No renal tubule tumours were observed in mice in the control group. The incidence of renal tubule adenoma or carcinoma (combined) in historical controls was 4/1366 (0.3 ± 0.7%; range, 0–2%), and only one renal tubule carcinoma was observed. [The Working Group noted that although there was no statistically significant increase, the renal tubule tumours may have been treatment-related because these tumours are rare in this strain of mouse.] Carcinomas of the small intestine occurred in three males at the highest dose and in one mouse in the control group. In addition, in the 15-month interim evaluation experiment, one carcinoma of the small intestine was observed in a male at the highest dose, but none were reported for the controls. In the historical

Table 3.1 Studies of carcinogenicity with CI Direct Blue 218 in experimental animals

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (M) 7 wk 104 wk NTP (1994)	Oral CI Direct Blue 218, copper complex of 3,3′-[(3,3′-dihydroxy[1,1′-biphenyl]-4,4′-diyl)bis(azo)] bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid] tetrasodium salt, 60%; reduction titration of azo groups indicated purity of 83%, and the authors stated that the titration estimate of purity was probably enhanced by the presence of reducible low molecular weight organic impurities containing the azo group as well as inorganic copper salts; 3,3′-dimethoxybenzidine, < 1 ppm; benzidine, ≤ 1 ppm Feed 0, 1000, 3000, 10 000 ppm 50, 50, 50, 50 44, 46, 42, 45	*Liver* Hepatocellular adenoma 16/50, 19/50, 17/50, 40/50* Hepatocellular carcinoma 7/50, 3/50, 8/50, 17/50* Hepatocellular adenoma or carcinoma (combined) 21/50, 20/50, 23/50, 45/50* *Kidney* Renal tubule adenoma 0/50, 2/50, 1/50, 1/50 Renal tubule carcinoma 0/50, 1/50, 0/50, 0/50 Renal tubule adenoma or carcinoma (combined) 0/50, 3/50, 1/50, 1/50 *Small intestine (jejunum)*: carcinoma 1/50, 0/50, 0/50, 3/50	 $P < 0.001$ (trend), *$P < 0.001$, all logistic regression tests $P < 0.001$ (trend), *$P = 0.019$, all logistic regression tests $P < 0.001$ (trend), *$P < 0.001$, all logistic regression tests NS NS NS NS	Principal strengths: complied with GLP; adequate duration of exposure and observation; used males and females Principal limitations: impurity of the test compound Incidence in historical controls: renal tubule adenoma or carcinoma (combined), 4/1366 (0.3 ± 0.7%; range, 0–2%); renal tubule adenoma, 3/1366 (0.2 ± 0.6%; range, 0–2%); renal tubule carcinoma, 1/1366 (0.1 ± 0.4%; range, 0–2%); small intestine adenoma, adenomatous polyp, or carcinoma (combined), 12/1374 (0.9 ± 1.0%; range, 0–4%); small intestine adenoma or adenomatous polyp, 5/1374 (0.4 ± 1.0%; range, 0–4%); small intestine carcinoma,7/1374 (0.5 ± 1.0%; range, 0–4%) Final mean body weight of group at the highest dose was 19% lower than that of control group

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (F) 7 wk 104 wk NTP (1994)	Oral CI Direct Blue 218, copper complex of 3,3′-[(3,3′-dihydroxy[1,1′-biphenyl]-4,4′-diyl)bis(azo)] bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid] tetrasodium salt, 60%; reduction titration of azo groups indicated purity of 83%, and the authors stated that the titration estimate of purity was probably enhanced by the presence of reducible low relative molecular mass organic impurities containing the azo group as well as inorganic copper salts; 3,3′-dimethoxybenzidine, < 1 ppm; benzidine, ≤ 1 ppm Feed 0, 1000, 3000, 10 000 ppm 49, 50, 49, 49 37, 40, 46, 38	*Liver* Hepatocellular adenoma 7/49, 12/50, 17/49*, 41/49** Hepatocellular carcinoma 5/49, 5/50, 6/49, 12/49 (24%) Hepatocellular adenoma or carcinoma (combined) 10/49, 15/50, 21/49*, 45/49**	$P < 0.001$ (trend), *$P = 0.041$, **$P < 0.001$, all logistic regression tests $P = 0.012$ (trend), logistic regression test $P < 0.001$ (trend), *$P = 0.045$, **$P < 0.001$, all logistic regression tests	Principal strengths: complied with GLP; adequate duration of exposure and observation; used males and females Principal limitations: impurity of the test compound Incidence in historical controls hepatocellular carcinoma, 80/1363 mice (range, 0–20%) Final mean body weight of the group at the highest dose was 27% lower than that of control group

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Rat, F344/N (M) 6–7 wk 103 wk NTP (1994)	Oral CI Direct Blue 218, copper complex of 3,3′-[(3,3′-dihydroxy[1,1′-biphenyl]-4,4′-diyl)bis(azo)] bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid] tetrasodium salt, 60%; reduction titration of azo groups indicated purity of 83%, and the authors stated that the titration estimate of purity was probably enhanced by the presence of reducible low molecular weight organic impurities containing the azo group as well as inorganic copper salts; 3,3′-dimethoxybenzidine, < 1 ppm; benzidine, ≤ 1 ppm Feed 0, 1000, 3000, 10 000 ppm 50, 50, 50, 51 30, 25, 29, 24	*Oral epithelium (pharynx)* Squamous cell papilloma 0/50, 0/50, 0/50, 5/50* Squamous cell carcinoma 0/50, 0/50, 0/50, 1/50 Squamous cell papilloma or carcinoma (combined) 0/50, 0/50, 0/50, 6/50* *Forestomach* Squamous cell papilloma 0/50, 0/50, 2/50, 1/50 Squamous cell carcinoma 0/50, 0/50, 1/50, 0/50 Squamous cell papilloma or carcinoma (combined) 0/50, 0/50, 3/50, 1/50	 $P < 0.001$ (trend), *$P = 0.026$, all logistic regression tests NS $P < 0.001$ (trend), *$P = 0.013$, all logistic regression tests NS NS NS	Principal strengths: complied with GLP; adequate duration of exposure and observation; used male and females Principal limitations: impurity of the test compound Incidence in historical controls: oral epithelium squamous cell papilloma or carcinoma (combined), 10/1253 (1.4 ± 0.8%; range, 0–4%); oral epithelium squamous cell papilloma, 10/1253 (1.4 ± 0.8%; range, 0–4%); oral epithelium squamous cell carcinoma, 0/1253; forestomach basal cell hyperplasia, significantly increased in treated groups; forestomach squamous cell papilloma or carcinoma (combined), 4/1253 (0.3 ± 0.8%; range, 0–2%); forestomach squamous cell papilloma, 3/1253 (0.2 ± 0.6%; range, 0–2%); forestomach squamous cell carcinoma (combined), 1/1253 (0.1 ± 0.4%; 0–2%) Final mean body weight of group at highest dose was 11% lower than that of control group

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Rat, F344/N (F) 6–7 wk 103 wk NTP (1994)	Oral CI Direct Blue 218, copper complex of 3,3′-[(3,3′-dihydroxy[1,1′-biphenyl]-4,4′-diyl)bis(azo)] bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid] tetrasodium salt, 60%; reduction titration of azo groups indicated purity of 83%, and the authors stated that the titration estimate of purity was probably enhanced by the presence of reducible low relative molecular mass organic impurities containing the azo group as well as inorganic copper salts; 3,3′-dimethoxybenzidine, < 1 ppm; benzidine, ≤ 1 ppm Feed 0, 1000, 3000, 10 000 ppm 51, 51, 50, 50 35, 29, 31, 25	*Uterus*: endometrial stromal polyp 1/50, 12/50*, 10/50**, 10/50***	*$P < 0.001$, **$P = 0.004$, ***$P = 0.001$, all logistic regression tests	Principal strengths: complied with GLP; adequate duration of exposure and observation; used males and females Principal limitations: impurity of the test compound Incidence in historical controls: endometrial stromal polyps, 205/1251 (16.4 ± 6.6%; range, 2–30%) Final mean body weight of group at the highest dose was 9% lower than that of control group
		Oral epithelium (pharynx): squamous cell papilloma 1/50, 1/50, 0/50, 2/50	NS	
		Forestomach: squamous cell papilloma 0/50, 0/50, 0/50, 1/50	NS	

F, female; GLP, Good Laboratory Practice; M, male; NS, not significant; ppm, parts per million; wk, week.

controls, the incidence of carcinoma of the small intestine was 7/1374 (0.5 ± 1.0%; range, 0–4%).

In female mice, there was a significant positive trend in the incidence of hepatocellular adenoma ($P < 0.001$, logistic regression trend test), hepatocellular carcinoma ($P = 0.012$, logistic regression trend test), and hepatocellular adenoma or carcinoma (combined) ($P < 0.001$, logistic regression trend test), with a significant increase in the incidence of hepatocellular adenoma (intermediate dose, $P = 0.041$; highest dose, $P < 0.001$; logistic regression tests) and hepatocellular adenoma or carcinoma (combined) (intermediate dose, $P = 0.045$; highest dose, $P < 0.001$; logistic regression tests).

Regarding non-neoplastic lesions, there was a significant increase in the incidence of eosinophilic foci of the liver at the highest dose in males, and a significant increase in the incidence of clear cell foci, eosinophilic foci, and foci (all) of the liver at the highest dose in females.

[The Working Group noted this was a well-conducted study that complied with GLP, males and females were used, and the duration of exposure and observation was adequate. The impurity of the compound used was a weakness of the study. The Working Group noted that, of the impurities, benzidine and 3,3′-dimethoxybenzidine were present at low levels.]

3.2 Rat

Oral administration (feed)

In a study of chronic toxicity and carcinogenicity that complied with GLP, four groups of 50–51 male and four groups of 50–51 female F344/N rats (age, 6–7 weeks) were given feed containing CI Direct Blue 218 at concentrations of 0, 1000, 3000, or 10 000 ppm (representing average daily doses of approximately 0, 40, 120, and 440 mg/kg bw per day for males, and 0, 50, 140, and 470 mg/kg bw per day for females, respectively) for the control group and groups at the lowest, intermediate, and highest dose, respectively, for 103 weeks (NTP, 1994). The agent was a desalted commercial dye of 60% copper complex of 3,3′-[(3,3′-dihydroxy[1,1′-biphenyl]-4,4′-diyl) bis(azo)]bis[5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid] tetrasodium salt; 11% water, 0.7% sodium chloride; reduction titration of azo groups indicated a purity of 83%, and the authors stated that the titration estimate of purity was probably "enhanced by the presence of reducible low molecular weight organic impurities containing the azo group as well as inorganic copper salts"; 3,3′-dimethoxybenzidine was < 1 ppm, and benzidine was ≤ 1 ppm. Survival of females at the highest dose was slightly reduced, but not significantly lower than that of the control group. At study termination, survival was 30/50, 25/50, 29/50, and 24/51 for males, and 35/51, 29/51, 31/50, and 25/50 for females for the control group and groups at the lowest, intermediate, and highest dose, respectively. Final mean body weights of rats at the highest dose was 11% lower than that of the controls for males, and 9% lower than that of the controls for females. Complete necropsies and full histopathological examinations were performed.

In male rats, there was a significant positive trend in the incidence of squamous cell papilloma of the oral epithelium (pharynx) ($P < 0.001$, logistic regression trend test), with a significant increase in incidence at the highest dose ($P = 0.026$, logistic regression test). In addition, one squamous cell carcinoma of the oral epithelium (pharynx) was also observed in one rat at the highest dose; no squamous cell carcinomas of the oral epithelium were observed in 1253 male historical controls. There was a significant positive trend in the incidence of squamous cell papilloma or carcinoma (combined) of the oral epithelium (pharynx) ($P < 0.001$, logistic regression trend test), with a significant increase in incidence at the highest dose ($P = 0.013$, logistic regression test). While there was no significant increase in the incidence of forestomach squamous cell

papilloma or carcinoma (combined), the incidence at the intermediate dose – control group, 0/50; lowest dose, 0/50; intermediate dose, 3/50 (6%); and highest dose, 1/50 (2%) – exceeded the upper bound of the range observed in historical controls in this laboratory (4/1253, 0.3 ± 0.8%; range, 0–2%). [Since the incidence of basal cell hyperplasia in the forestomach was significantly increased at the two higher doses in males, and forestomach tumours are rare in this strain of rat, the Working Group considered that the increased incidence of forestomach tumours may have been treatment-related.]

In female rats, the incidence of uterine endometrial stromal polyps – control group, 1/50 (2%); lowest dose, 12/50 (24%); intermediate dose, 10/50 (20%); highest dose, 10/50 (20%) – was significantly increased in each treated group compared with controls (lowest dose, $P < 0.001$; intermediate dose, $P = 0.004$; highest dose, $P = 0.001$, logistic regression test). However, there was no dose–response relationship and, when compared with the incidence and range in historical controls (205/1251, 16.4 ± 6.6%; range, 2–30%), the incidence in the concurrent controls (1/50, 2%) was very low. [The Working Group considered that the higher incidence of endometrial stromal polyps in the exposed groups was probably not treatment-related.]

Regarding non-neoplastic lesions, there was a significant increase in the incidence of basal cell hyperplasia of the forestomach in males at the intermediate and highest dose.

[The Working Group noted this was a well-conducted study that complied with GLP, males and females were used, and the duration of exposure and observation was adequate. The impurity of the compound used was a weakness of the study. The Working Group noted that, of the impurities, benzidine and 3,3′-dimethoxybenzidine were present at low levels.]

3.3 Evidence synthesis for cancer in experimental animals

The carcinogenicity of CI Direct Blue 218 has been assessed in one study in male and female mice and in one study in male and female rats exposed by oral administration (in the feed).

In a study that complied with GLP, male and female B6C3F$_1$ mice were treated with CI Direct Blue 218 in the feed (NTP, 1994). In male mice, there was a significant positive trend and significant increase in the incidence of hepatocellular adenoma, hepatocellular carcinoma, and hepatocellular adenoma or carcinoma (combined). In female mice, there was a significant positive trend in the incidence of hepatocellular adenoma, hepatocellular carcinoma, and hepatocellular adenoma or carcinoma (combined), with a significant increase in the incidence of hepatocellular adenoma and hepatocellular adenoma or carcinoma (combined).

In a study that complied with GLP, male and female F344/N rats were treated with CI Direct Blue 218 in the feed (NTP, 1994). In male rats, there was a significant positive trend and significant increase in the incidence of squamous cell papilloma of the oral epithelium (pharynx), and squamous cell papilloma or carcinoma (combined) of the oral epithelium (pharynx). In female rats, a statistically significant increase in the incidence of uterine endometrial stromal polyps was probably not treatment-related.

4. Mechanistic Evidence

4.1 Absorption, distribution, metabolism, and excretion

No data on the absorption, distribution, metabolism, or excretion of CI Direct Blue 218 in mammalian systems were available to the Working Group. [Based on data from bacterial

mutagenicity assays (see Section 4.2.1(b)(iii)), the Working Group noted that the azo bond of CI Direct Blue 218 could be reduced, generating 3,3′-dihydroxybenzidine or its respective copper-complexed form.]

4.2 Evidence relevant to key characteristics of carcinogens

This section summarizes the evidence for the key characteristics of carcinogens (Smith et al., 2016), including whether CI Direct Blue 218 is genotoxic or causes immortalization. Insufficient data were available for the evaluation of other key characteristics of carcinogens. No data relevant to the key characteristics of carcinogens were available from the Toxicology in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) research programmes in the USA because CI Direct Blue 218 was not tested in this assay battery.

4.2.1 Is genotoxic

(a) Humans

No data were available to the Working Group.

(b) Experimental systems

(i) Non-human mammals in vivo

No data were available to the Working Group.

(ii) Non-human mammals in vitro

See Table 4.1.

CI Direct Blue 218 induced mutations in the presence, but not in the absence, of metabolic activation in a mouse lymphoma assay (Mitchell et al., 1997). Negative results were obtained when the ability of CI Direct Blue 218 to induce chromosomal aberrations was tested in Chinese hamster ovary cells with and without metabolic activation (NTP, 2020). It significantly increased the frequency of sister-chromatid exchanges compared with the negative control without, but not with, metabolic activation. [The Working Group noted that no information on purity was provided in these studies.]

(iii) Non-mammalian experimental systems

See Table 4.2.

Woodruff et al. (1985) reported that CI Direct Blue 218 did not induce sex-linked recessive mutation in meiotic and postmeiotic germ cell stages of Canton-S male *Drosophila melanogaster* treated with CI Direct Blue 218 in the feed or via injection. [The Working Group noted that no information on purity was provided.]

Results were largely negative in the available *Salmonella*/microsome mutagenicity assays. Gregory et al. (1981) reported negative results in tests performed under different reductive conditions (addition of riboflavin, nitrogen gas, and sodium dithionate) in the presence of metabolic activation in strains TA98 and TA100. In tests performed in the presence of sodium dithionate in strain TA100, more than double the numbers of revertants per plate in comparison with the negative control were obtained at the three lowest doses tested, whereas results at the two highest doses were comparable with those for the negative controls. [The Working Group noted that the experiment was not repeated, so no conclusion on the mutagenicity of CI Direct Blue 218 in TA100 could be drawn. No information on purity was provided.]

Prival et al. (1984) reported negative results for CI Direct Blue 218 (commercial samples) [the Working Group noted that no information on purity was provided] in the TA98 strain in the presence of metabolic activation (hamster liver S9), with and without modifications to promote the reduction of the azo bond (Prival & Mitchell, 1982). These modifications consisted of a 30-minute preincubation without agitation (to reduce oxygen) and the addition of flavin mononucleotide. *ortho*-Dianisidine (3,3′-dimethoxybenzidine), the parental aromatic amine of CI Direct Blue 218, was tested for mutagenicity

Table 4.1 Genetic and related effects of CI Direct Blue 218 in non-human mammals in vitro

End-point	Species, tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Gene mutation, *Tk* locus	Mouse, lymphoma L5178Y/*Tk*$^{+/-}$	–	+	40 µg/mL	Purity, NR	Mitchell et al. (1997)
Chromosomal aberrations	Chinese hamster, ovary (CHO) cells	–	–	500 µg/mL	Purity, NR	NTP (2020)
Sister-chromatid exchange	Chinese hamster, ovary (CHO) cells	+	–	200 µg/mL	Purity, NR	NTP (2020)

HIC, highest ineffective concentration; LEC, lowest effective concentration; NR, not reported; *Tk*, thymidine kinase.
[a] +, positive; –, negative.

Table 4.2 Genetic and related effects of CI Direct Blue 218 in non-mammalian experimental systems

Test system (species, strain)	End-point	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Drosophila melanogaster	Sex-linked recessive mutation	–	NA	10 000 µg/mL (feed);1000 µg/mL (injected)	Purity, NR; single concentrations	Woodruff et al. (1985)
Salmonella typhimurium TA98, TA100	Reverse mutation	NT	–	1000 µg/plate	Purity, NR; tests under reductive conditions with and without riboflavin	Gregory et al. (1981)
Salmonella typhimurium TA98	Reverse mutation	NT	–	1000 µg/plate	Purity, NR; S9 10%; tests under reductive conditions with sodium dithionate	Gregory et al. (1981)
Salmonella typhimurium TA100	Reverse mutation	NT	Inconclusive	1000 µg/plate	Purity, NR; S9 10%; tests under reductive conditions with sodium dithionate	Gregory et al. (1981)
Salmonella typhimurium TA98, TA100, TA1537	Reverse mutation	NT	–	1091.90 µg/plate	Purity, NR; S9 10%; tests under reductive conditions with and without flavin mononucleotide	Prival et al. (1984)
Salmonella typhimurium TA1538	Reverse mutation	NT	–	545.9 µg/plate	Purity, NR; S9 30%; tests under reductive conditions in two protocols; (a) with and without rat caecal bacteria and (b) with flavin mononucleotide and S9 30%	Reid et al. (1984)
Salmonella typhimurium TA1535, TA1537, TA97, TA98, TA100	Reverse mutation	–	–	10 000 µg/plate	Purity, 44.8%; S9 10%; tests under oxidative conditions	Mortelmans et al. (1986)

HIC, highest ineffective concentration; LEC, lowest effective concentration; NA, not applicable; NR, not reported; NT, not tested; S9, 9000 × *g* supernatant.
[a] –, negative.

under the same conditions and provided a clear positive response that was unchanged by the addition of CI Direct Blue 218. [The Working Group noted that this could indicate that the dye did not release *ortho*-dianisidine (3,3′-dimethoxybenzidine) after incubation with S9, because if this was the case, the test with the dye would provide a mutagenic response. In fact, the cleavage of the azo bond of CI Direct Blue 218 would generate 3,3′-dihydroxybenzidine, which gives negative results when tested with S9 at 10%. 3,3′-Dihydroxybenzidine is only mutagenic when S9 is present at 30% (NTP, 2018).] Prival et al. (1984) also reported negative results for CI Direct Blue 218 in strains TA100 and TA1537, and confirmed the negative results for TA98 using rat liver S9.

Reid et al. (1984) reported negative results for a commercial sample of CI Direct Blue 218 in strain TA1538 with endogenous metabolic activation. They tested the dye with and without incubation with a washed suspension of rat caecal bacteria (caecal reduction system) to promote reduction of the azo bond, simulating intestinal metabolism. The assay was also performed with flavin mononucleotide and Syrian golden hamster S9 using the protocol described by Prival & Mitchell (1982). [The Working Group noted that no information on purity was provided.]

Mortelmans et al. (1986) reported negative results for CI Direct Blue 218 (purity, 44.8%) in strains TA1535, TA1537, TA97, TA98, and TA100, with and without endogenous metabolic activation and preincubation. [The Working Group noted that the test was performed under oxidative conditions.]

[The Working Group noted that although the available *Salmonella* tests were performed with the five strains recommended by the Organisation for Economic Co-operation and Development (OECD, 2020), CI Direct Blue 218 was not tested in one of the strains sensitive to aromatic amines, e.g. YG1041 (Hagiwara et al., 1993). Zwarg et al. (2018) and Umbuzeiro et al. (2021) have shown the importance of including this strain to detect the mutagenicity of azo dyes containing NH_2 radicals, which is the case with CI Direct Blue 218.]

4.2.2 Causes immortalization

Matthews et al. (1993) reported equivocal results for commercial CI Direct Blue 218 in a study with the objective of introducing an improved method for detecting chemically induced morphological transformation of A31-1-13 BALB/c-3T3 cells. CI Direct Blue 218 was tested at six concentrations (125–2000 µM). No concentration–response relationship was observed, but at 250 and 500 µM, significant differences were observed in comparison with the negative control. [The Working Group noted that no information on purity was provided.]

4.3 Data relevant to comparisons across agents and end-points

CI Direct Blue 218 was not tested in biochemical and cell-based assays run by the United States Environmental Protection Agency (US EPA) and the United States National Institutes of Health Toxicity Forecaster/Toxicology in the 21st Century (ToxCast/Tox21) high-throughput screening programmes (Chiu et al., 2018; Guyton et al., 2018); see the monograph on gentian violet and leucogentian violet in the present volume for more details.

5. Summary of Data Reported

5.1 Exposure characterization

Colour Index (CI) Direct Blue 218 is a copper-chelated dimethoxybenzidine-based azo dye. It is used as a dye for cellulose, acetate, nylon, silk, wool, tissue and fine papers, and textile goods with a urea-formaldehyde finish.

Only one environmental study on CI Direct Blue 218 was identified, and this did not detect the dye in river water, sediments, or fish downstream of textile mills in a Canadian river. In general, dyes and pigments derived from benzidine and its congeners may be released into environmental waters as a constituent of industrial process waters, into the atmosphere by industrial sources, or via the disposal of dyed articles. Azo dyes are persistent bioaccumulative substances and, therefore, may contaminate groundwater, agricultural fields, aquatic plants, and fish.

The potential for occupational exposure to CI Direct Blue 218 occurs during the manufacturing process, from the application of the dye on products, and from further processing of dyed products that results in particle formation. Potential exposure routes include inhalation of dust or mist, accidental ingestion, and direct contact with the skin. No data on occupational exposure levels were identified.

No data on exposure to CI Direct Blue 218 in the general population have been identified; however, most environmental exposure to benzidine-based dyes has been described to be through contact with contaminated air, water, or soil. Benzidine-based dyes in finished products are not considered to migrate as a result of washing, or via perspiration or saliva.

Few specific regulations or guidelines for CI Direct Blue 218 exist, but there are guidelines for dimethoxybenzidine-based azo dyes and for azo dyes that are known to be metabolized to aromatic amines.

5.2 Cancer in humans

No data were available to the Working Group.

5.3 Cancer in experimental animals

Exposure to CI Direct Blue 218 caused an increase in the incidence of malignant neoplasms in both sexes of a single species (mouse) in a study that complied with Good Laboratory Practice (GLP), and an increase in the incidence of an appropriate combination of benign and malignant neoplasms in one sex of another species in a study that complied with GLP.

In $B6C3F_1$ mice exposed to CI Direct Blue 218 in the feed, there was a significant positive trend and significant increase in the incidence of hepatocellular carcinoma in males, and a significant positive trend in the incidence of hepatocellular carcinoma in females. In another species (rat), CI Direct Blue 218 in the feed significantly increased (with a positive trend and by pairwise comparisons) the incidence of squamous cell papilloma or carcinoma (combined) of the oral epithelium (pharynx) in F344/N male rats in a study that complied with GLP.

5.4 Mechanistic evidence

No data on absorption, distribution, metabolism, or excretion were available.

Few mechanistic data were available. CI Direct Blue 218 was mutagenic in one study in non-human mammals in a test in vitro, the mouse lymphoma assay, in the presence of metabolic activation. In Chinese hamster ovary cells, CI Direct Blue 218 induced sister-chromatid exchange only in the absence of metabolic activation but did not induce chromosomal aberrations in the presence or absence of metabolic activation. CI Direct Blue 218 was not mutagenic in *Drosophila melanogaster* and gave negative results in the available Ames tests, which did not cover a wide range of doses and strains.

No data relevant to the key characteristics of carcinogens were available from the Toxicology in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) research programmes in the USA

because CI Direct Blue 218 was not tested in this assay battery.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *inadequate evidence* in humans regarding the carcinogenicity of CI Direct Blue 218.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of CI Direct Blue 218.

6.3 Mechanistic evidence

There is *inadequate mechanistic evidence*.

6.4 Overall evaluation

CI Direct Blue 218 is *possibly carcinogenic to humans (Group 2B)*.

6.5 Rationale

The *Group 2B* evaluation for CI Direct Blue 218 is based on *sufficient evidence* for cancer in experimental animals. The evidence regarding cancer in humans is *inadequate* as no studies were available. The mechanistic evidence is *inadequate* for CI Direct Blue 218. The *sufficient evidence* for cancer in experimental animals is based on an increase in the incidence of malignant neoplasms in males and females of a single species in a study that complies with GLP, and an increase in the incidence of an appropriate combination of benign and malignant neoplasms in males of another species in another study that complies with GLP.

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LIST OF ABBREVIATIONS

APCI-MS	atmospheric pressure chemical ionization–mass spectrometry
bw	body weight
CAS	Chemical Abstracts Service
CAT	catalase
CHO	Chinese hamster ovary
CI	Colour Index
DMSE	diphenylmethyl selenocyanate
DNA	deoxyribonucleic acid
EC	European Community
EFSA	European Food Safety Authority
EU	European Union
GC	gas chromatography
GLP	Good Laboratory Practice
Gpt	glutamic-pyruvate transaminase
GSH	glutathione
γ-H2AX	phosphorylated histone H2AX
HPLC	high-performance liquid chromatography
Hprt	hypoxanthine-guanine phosphoribosyltransferase
IC_{50}	half-maximal inhibition
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
JECFA	Joint Food and Agriculture Organization of the United Nations/World Health Organization Expert Committee on Food Additives
LC	liquid chromatography
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
MS	mass spectrometry
NIOSH	National Institute for Occupational Health and Safety
NTP	National Toxicology Program
OSHA	Occupational Safety and Health Administration
ppm	parts per million
QuEChERS	quick easy cheap effective rugged safe
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals

SCCNFP	Scientific Committee on Cosmetic and Non-Food Products intended for Consumers
SHE	Syrian hamster embryo
SOD	superoxide dismutase
T3	triiodothyronine
T4	thyroxine
Tox21	Toxicology in the 21st Century programme
ToxCast	Toxicity Forecaster programme
TPO	thyroid peroxidase
TSH	thyroid-stimulating hormone
US EPA	United States Environmental Protection Agency
UV	ultraviolet
w/w	weight per weight

ANNEX 1. SUPPLEMENTARY MATERIAL FOR SECTION 4, MECHANISTIC EVIDENCE

Of the compounds included in *IARC Monographs* Volume 129, four (gentian violet, malachite green chloride, malachite green oxalate, and leucomalachite green) have been evaluated in at least some of the high-throughput screening assays performed by the United States Environmental Protection Agency and the United States National Institutes of Health (US EPA, 2020a, b, c, d).

Details of the specific assays and end-points available for each chemical and the mapping to the key characteristics of carcinogens are available in the file available at: https://www.publications.iarc.fr/603.

Please report any errors to imo@iarc.fr.

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SUMMARY OF FINAL EVALUATIONS

Summary of final evaluations for Volume 129

Agent	Evidence stream			Overall evaluation
	Cancer in humans	Cancer in experimental animals	Mechanistic evidence	
Gentian violet	*Inadequate*	*Sufficient*	*Limited*	Group 2B
Leucogentian violet	*Inadequate*	*Inadequate*	*Inadequate*	Group 3
Malachite green	*Inadequate*	*Limited*	*Limited*	Group 3
Leucomalachite green	*Inadequate*	*Sufficient*	*Limited*	Group 2B
CI Direct Blue 218	*Inadequate*	*Sufficient*	*Inadequate*	Group 2B

IARC MONOGRAPHS

This volume of the *IARC Monographs* provides evaluations of the carcinogenicity of three dyes and their two leucometabolites: gentian violet, leucogentian violet, malachite green, leucomalachite green, and CI Direct Blue 218.

Gentian violet and malachite green are cationic triphenylmethane dyes widely used for textiles, paper, and acrylic products, as biological stains, and in some hair dyes and other cosmetics. Because of their antibacterial and antifungal properties, they have had various medical, veterinary, and aquaculture applications, including the treatment of livestock, animal feed, ornamental fish, and farmed fish and shellfish.

Leucogentian violet and leucomalachite green are used as precursors in the production of their parent compounds and have direct applications as chromogenic reagents in analytical chemistry and as radiochromic indicators in dosimeters.

CI Direct Blue 218 is a copper-chelated dimethoxybenzidine-based azo dye used for cellulose, acetate, nylon, silk, wool, tissue, fine papers, and textile goods.

For all agents, data were sparse regarding exposure levels, but indicated that exposures can occur in occupational settings and in the general population.

An *IARC Monographs* Working Group reviewed evidence from cancer bioassays in experimental animals and mechanistic studies to assess the carcinogenic hazard to humans of exposure to these agents and concluded that:

- Gentian violet, leucomalachite green, and CI Direct Blue 218 are *possibly carcinogenic to humans (Group 2B)*
- Leucogentian violet and malachite green are *not classifiable as to their carcinogenicity to humans (Group 3).*

IARC MONOGRAPHS ANNIVERSARY 2021