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International Agency for Research on Cancer

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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)

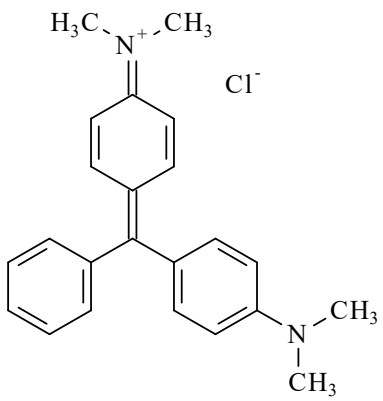
Chem. Abstr. Serv. name: malachite green (American Chemical Society, 2021a); methanaminium,*N*-[4-[[4-(dimethylamino)phenyl]phenylmethylene]-2,5-cyclohexadien-1-ylidene]-*N*-methyl-chloride (1:1) (ECHA, 2020a)

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)

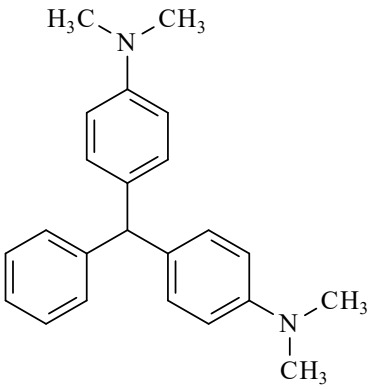
EC No.: 204-961-9

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).

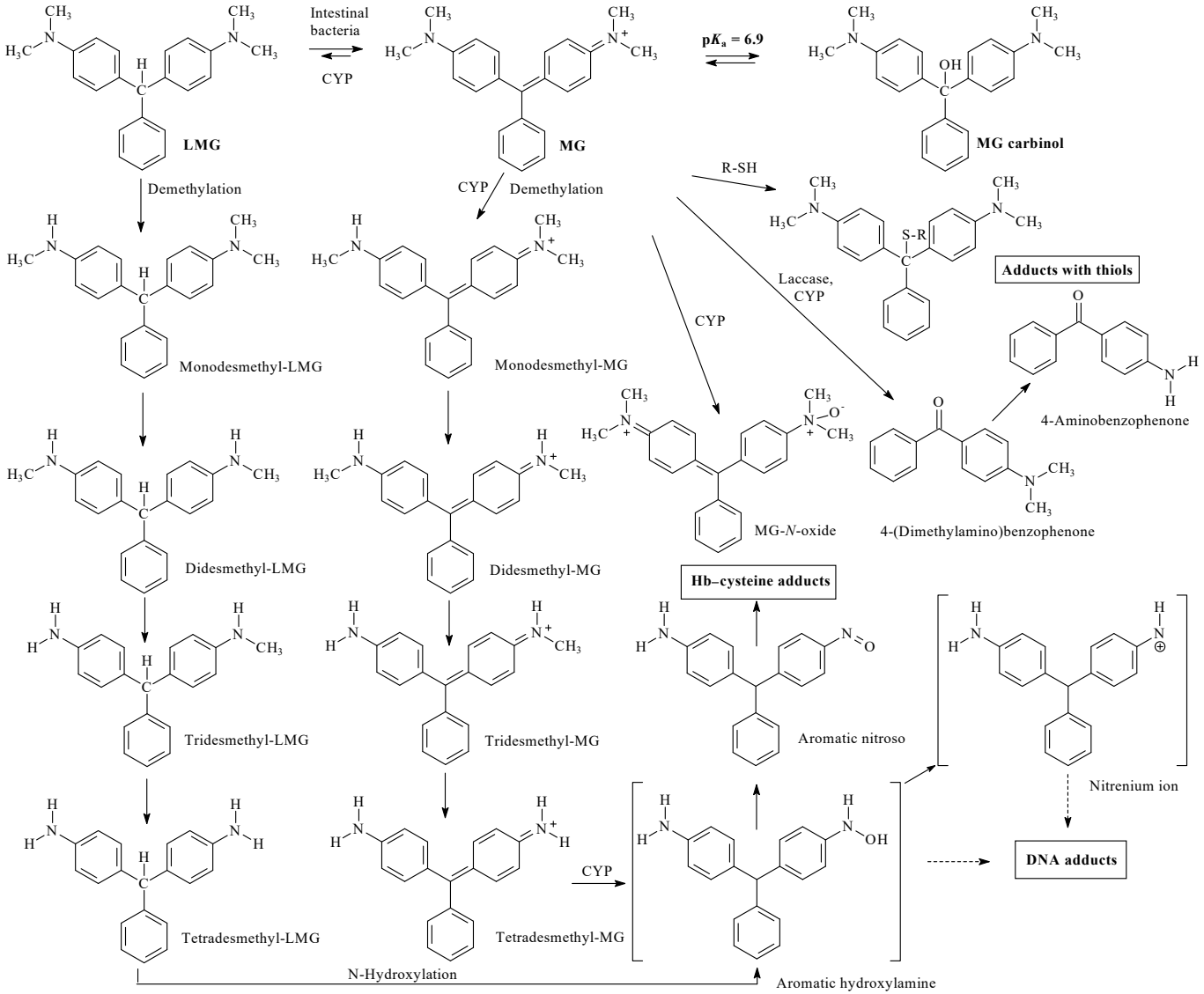
After intravenous injection of malachite green in rats, leucomalachite green was detected in the liver, kidney, heart, lung, and muscle after 2 hours (reviewed in NTP, 2005), which suggests that malachite green can be reduced to leucomalachite green in rat tissues (Werth & Boiteux, 1968). In cultures 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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