

# METHYLENE BLUE

## 1. Exposure Data

Methylene blue was originally synthesized in 1876 as an aniline-based dye for the textile industry ([Berneth, 2008](#)), but scientists such as Robert Koch and Paul Ehrlich were quick to realize its potential for use in microscopy stains ([Ehrlich, 1881](#); [Oz et al., 2011](#)). The observation of selective staining and inactivation of microbial species led to the testing of aniline-based dyes against tropical diseases ([Oz et al., 2011](#)). Methylene blue was the first such compound to be administered to humans, and was shown to be effective in the treatment of malaria ([Guttman & Ehrlich, 1891](#); [Oz et al., 2011](#)). Methylene blue was also the first synthetic compound ever used as an antiseptic in clinical therapy, and the first antiseptic dye to be used therapeutically. In fact, the use of methylene blue and its derivatives was widespread before the advent of sulfonamides and penicillin ([Oz et al., 2011](#)).

### 1.1 Chemical and physical data

#### 1.1.1 Nomenclature

*Chem. Abstr. Serv. Reg. No.:* 61-73-4 (anhydrous); 7220-79-3 (methylene blue trihydrate)  
According to recent research, methylene blue occurs in the form of several different hydrates, but not as trihydrate ([Rager et al., 2012](#)). [The Working Group noted that most of the scientific literature refers only to

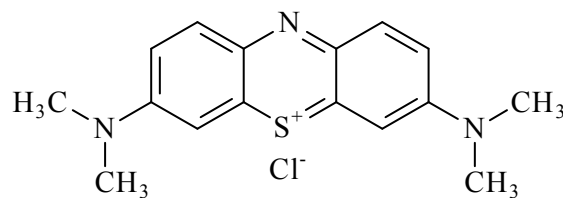
“methylene blue” independent of hydration state. Due to its hygroscopic nature, commercial methylene blue is typically sold as the hydrate, but is sometimes incorrectly presented as the trihydrate.]

*Chem. Abstr. Serv. Name:* Phenothiazin-5-ium, 3,7-bis(dimethylamino)-, chloride ([O’Neil et al., 2006](#))

*IUPAC Systematic Name:* [7-(Dimethylamino)phenothiazin-3-ylidene]-dimethylazanium chloride ([PubChem, 2013](#))

*Synonyms:* Aizen methylene blue; Basic blue 9 (8CI); Calcozine blue ZF; Chromosmon; C.I. 52 015; Methylthionine chloride; Methylthioninium chloride; Phenothiazine-5-ium,3,7-bis, (dimethylamino)-, chloride; Swiss blue; Tetramethylene blue; Tetramethylthionine chloride ([NTP, 2008](#); [PubChem, 2013](#)).

#### 1.1.2 Structural and molecular formulae and relative molecular mass



Relative molecular mass (anhydrous form): 319.85 ([PubChem, 2013](#))

### 1.1.3 Chemical and physical properties of the pure substance

*Description:* Dark green crystals or crystalline powder with bronze lustre, odourless, stable in air, deep blue solution in water or alcohol, forms double salts ([PubChem, 2013](#))

*Melting point:* 100–110 °C (decomposition) ([PubChem, 2013](#))

*Density:* 1.0 g/mL at 20 °C ([ChemNet, 2013](#))

*Solubility:* 43.6 g/L in water at 25 °C; also soluble in ethanol ([PubChem, 2013](#))

*Vapour pressure:*  $1.30 \times 10^{-7}$  mm Hg at 25 °C (estimated) ([PubChem, 2013](#)).

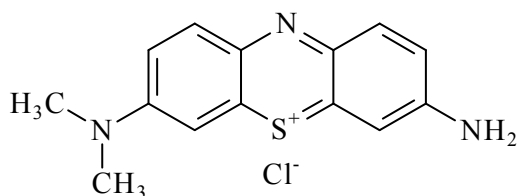
### 1.1.4 Technical products and impurities

#### (a) Trade names

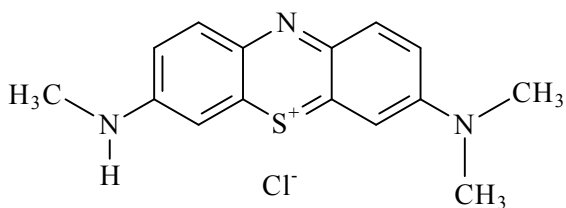
Desmoid piller; desmoidpillen; panatone; urolene blue; vitableu ([NTP, 2008](#))

#### (b) Impurities

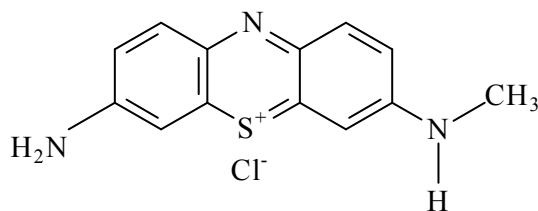
- 3-Amino-7-(dimethylamino)phenothiazin-5-ium chloride (azure A) ([PubChem, 2013](#))



- 3-(Dimethylamino)-7-(methylamino)phenothiazin-5-ium chloride or *N,N,N'*-trimethylthionin (azure B) ([PubChem, 2013](#))



- 3-(Amino)-7-(methylamino)phenothiazin-5-ium chloride (azure C) ([PubChem, 2013](#))



## 1.2 Analysis

There are several compendial and non-compendial methods for the analysis of methylene blue ([Table 1.1](#)). To quantify methylene blue in formulations, ultraviolet-visible spectroscopy can be conducted. For the quantification of methylene blue in biological specimens, liquid chromatography coupled with different detectors seems to be the method of choice.

## 1.3 Production and use

### 1.3.1 Production

Methylene blue is synthesized commercially by oxidation of *N,N*-dimethyl-phenylenediamine with sodium dichromate ( $\text{Na}_2\text{Cr}_2\text{O}_7$ ) in the presence of sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_3$ ), followed by further oxidation in the presence of *N,N*-dimethylaniline ([NTP, 2008](#)). Methylene blue hydrochloride is isolated by addition of 30% hydrochloric acid and of a saturated common salt solution to the dye solution; after filtration, the product is washed with a 2% common salt solution. Instead of sodium dichromate, manganese dioxide, and catalytic amounts of copper sulfate can be used for the oxidation ([Berneth, 2008](#)).

Methylene blue of high purity can be obtained by chloroform extraction of impurities from solutions of raw dye in borate buffer at pH 9.5–10, followed by acidification of the aqueous solution and isolation of the dye ([Berneth, 2008](#)).

Table 1.1 Some compendial and non-compendial methods for the analysis of methylene blue

Matrix	Sample preparation	Assay method	Detection limit	Reference
<i>Compendial methods</i>				
Assay	-	UV-visible spectroscopy Wavelength: 663 nm	-	<a href="#">US Pharmacopeial Convention (2013)</a>
Assay	-	Iodimetric titration Titration with sodium thiosulfate using starch solution as indicator	-	<a href="#">British Pharmacopoeia Commission (2005)</a>
Related substance test	-	LC-UV Column: C <sub>18</sub> Mobile phase: acetonitrile and phosphoric acid (3.4 mL in 1000 mL of water) (27 : 73, v/v) Flow rate: 1 mL/min Wavelength: 246 nm	-	<a href="#">British Pharmacopoeia Commission (2005)</a>
<i>Non-compendial methods</i>				
Human blood	Addition of NaCl and dichloroethane, centrifugation, analysis of dichloroethane layer	UV-visible spectroscopy Wavelength: 660 nm	0.02 µg/mL (LOD)	<a href="#">DiSanto &amp; Wagner (1972)</a>
Human urine	Addition of NaCl and dichloroethane, centrifugation, analysis of dichloroethane layer	UV-visible spectroscopy Wavelength: 660 nm	0.02 µg/mL (LOD)	<a href="#">DiSanto &amp; Wagner (1972)</a>
Rat tissue	Blotting on filter paper, addition of 0.1 N hydrochloric acid, homogenization, addition of NaCl and dichloroethane, centrifugation, analysis of dichloroethane layer	UV-visible spectroscopy Wavelength: 660 nm	0.02 µg/mL (LOD)	<a href="#">DiSanto &amp; Wagner (1972)</a>
Human blood	Haemolysis, addition of sodium hexanesulfonate, extraction (dichloroethane), centrifugation, analysis of organic layer	UV-visible spectroscopy Wavelength: 657 nm	0.1 µg/mL (LOQ)	<a href="#">Belaz-David et al. (1997)</a>
Human plasma	Addition of sodium hexanesulfonate, extraction (dichloroethane), centrifugation, analysis of organic layer	UV-visible spectroscopy Wavelength: 657 nm	0.1 µg/mL (LOQ)	<a href="#">Belaz-David et al. (1997)</a>
Human urine	Reduction of leucomethylene blue into methylene blue, addition of sodium hexanesulfonate, extraction (dichloroethane), centrifugation, analysis of organic layer	UV-visible spectroscopy Wavelength: 657 nm	3 µg/mL (LOQ)	<a href="#">Belaz-David et al. (1997)</a>

**Table 1.1 (continued)**

Matrix	Sample preparation	Assay method	Detection limit	Reference
Human blood	Mixing with sodium hexanesulfonate, extraction (dichloroethane), centrifugation, evaporation	LC-UV Column: cyano Mobile phase: ammonium dihydrogen phosphate, acetonitrile and methanol pH 2.75 Flow rate: 0.7 mL/min Wavelength: 660 nm	9 nmol/L (LOQ)	<a href="#">Peter et al. (2000)</a>
Human urine	Reduction of leucomethylene blue into methylene blue, mixing with sodium hexanesulfonate, extraction (dichloroethane), centrifugation, evaporation	LC-UV Column: cyano Mobile phase: ammonium dihydrogen phosphate, acetonitrile and methanol pH 2.75 Flow rate: 0.7 mL/min Wavelength: 660 nm	9 nmol/L (LOQ)	<a href="#">Peter et al. (2000)</a>
Human blood and plasma	Precipitation with acetonitrile, centrifugation, and analysis of clear supernatant	LC-ESI-MS Column: C <sub>18</sub> Mobile phase: 0.1% acetic acid in 5 mM acetate buffer and acetonitrile Flow rate: 0.35 mL/min	0.5 ng/mL (LOQ)	<a href="#">Rengelshausen et al. (2004)</a>
Human blood and plasma	Acidic protein precipitation, centrifugation, analysis of clear supernatant	IEX-MS Column: upisphere mixed mode Mobile phase: 0.1% acetic acid including 100 mM ammonium acetate (solvent A) and 2.5% formic acid/acetonitrile (1 : 1, v/v) including 500 mM ammonium acetate (solvent B) Flow rate: 0.45 mL/min	75 ng/mL (LOQ)	<a href="#">Burhenne et al. (2008)</a>
Dried blood	Cutting of paper sheet, soaking in demineralized water, ultrasonication, protein precipitation, and analysis of clear supernatant	IEX-MS Column: upisphere mixed mode Mobile phase: 0.1% acetic acid including 100 mM ammonium acetate (solvent A) and 2.5% formic acid/acetonitrile (1 : 1, v/v) including 500 mM ammonium acetate (solvent B) Flow rate: 0.45 mL/min	75 ng/mL (LOQ)	<a href="#">Burhenne et al. (2008)</a>
Human urine	Dilution of urine	FIA-PIF Wavelength: $\lambda_{\text{ex}}$ at 345 nm and $\lambda_{\text{em}}$ at 485 nm pH 13 Flow rate: 2 mL/min	16 ng/mL (LOD) 0.06 $\mu\text{g/mL}$ (LOQ)	<a href="#">Laassis et al. (1994)</a>

Table 1.1 (continued)

Matrix	Sample preparation	Assay method	Detection limit	Reference
Human urine	Addition of sodium hexanesulfonate, extraction (dichloromethane), evaporation, reconstitution in water	CE-UV Extended light path (bubble) capillary Mobile phase: 100 mM phosphate buffer with 25% acetonitrile pH 2.5 Wavelength: 292 and 592 nm	1 µg/mL (LOQ)	<a href="#">Borwitzky et al. (2005)</a>
Rat urine and mouse urine	Addition of <i>p</i> -toluene sulfonic acid, mixing, addition of dichloroethane, centrifugation, collection of dichloroethane layer, evaporation, reconstitution in 0.1% trifluoroacetic acid and acetonitrile	LC-UV Column: C <sub>18</sub> Mobile phase: acetonitrile and 0.1% trifluoroacetic acid in water pH adjusted to ~2.74 with triethylamine Flow rate: 1 mL/min Wavelength: 660 nm	3.9 ng/mL (LOD) 13 ng/mL (LOQ)	<a href="#">Gaudette &amp; Lodge (2005)</a>
Rat blood	Addition of <i>p</i> -toluene sulfonic acid, buffering at pH 3 with ammonium acetate buffer, addition of acetonitrile and ultrasonic extraction, defatting of liquid phase with hexane, addition of dichloromethane, centrifugation, evaporation, reconstitution in water	CE-ESI-MS Fused silica capillary Electrolyte: 2 mol/L acetic acid Sheath liquid: methanol : water (80 : 20, v/v)	0.22 µg/mL (LOD) 0.5 µg/mL (LOQ)	<a href="#">Yang et al. (2011)</a>
Cows' milk	Addition of acetonitrile, centrifugation, transferring of liquid into separating funnel, addition of NaCl, extraction with chloroform twice, collection of lower layer, evaporation, dissolve in acetonitrile, column clean-up with CBA column, evaporation of eluent, reconstitution in methanol	LC-UV Column: cyano Mobile phase: acetonitrile and acetate buffer pH 4.5 Flow rate: 1 mL/min Wavelength: 627 nm	2.5 ppb [ng/mL] (LOD) 5 ppb [ng/mL] (LOQ)	<a href="#">Munns et al. (1992)</a>
Muscle of fish (rainbow trout)	Addition of McIlvaine buffer (pH 3.0), homogenization, addition of acetonitrile, centrifugation, washing of supernatant with <i>n</i> -hexane twice, addition of 10% NaCl solution and dichloromethane, addition of sodium sulfate to dichloromethane layer, filtration, evaporation, reconstitution with methanol	LC-UV Column: C <sub>18</sub> Mobile phase: 0.1 M citrate buffer, acetonitrile pH 3.0 Flow rate: 0.8 mL/min Wavelength: 636 nm	3 µg/kg (LOD)	<a href="#">Kasuga et al. (1991)</a>

Table 1.1 (continued)

Matrix	Sample preparation	Assay method	Detection limit	Reference
Fish tissue	Homogenization with ammonium acetate (pH 4.5) and acetonitrile, addition of basic aluminium oxide, centrifugation, transferring of supernatant into separating funnel, re-extraction of solid residue in the same manner, further extraction (dichloromethane), addition of DDQ and formic acid to dichloromethane layer, clean-up with isolate strong cation-exchange cartridge	LC-ESI-MS Column: C <sub>18</sub> Mobile phase: ammonium acetate and acetonitrile pH 4.5 Flow rate: 0.3 mL/min	23.8 µg/kg (LOD)	<a href="#">Tarbin et al. (2008)</a>
Edible aquatic products (eel, shrimp)	Addition of <i>p</i> -toluene sulfonic acid, buffering at pH 4.5 with sodium acetate buffer, extraction (acetonitrile, dichloromethane and diglycol), centrifugation, evaporation, reconstitution in acetonitrile, clean-up with neutral alumina and weak cation-exchange cartridges, evaporation, reconstitution in 3 : 7 (v/v) methanol : water solution	LC-ESI-MS Column: C <sub>18</sub> Mobile phase: methanol, 0.1% formic acid pH 4.5 Flow rate: 250 µL/mL	0.1 µg/kg (LOD) 0.5 µg/kg (LOQ)	<a href="#">Xu et al. (2009)</a>
Formulation	–	LC-ED Column: cyano Mobile phase: methanol, 0.1 M sodium acetate pH 4.5 Flow rate: 0.8 mL/min First derivative UV spectroscopy Wavelength: 273 nm	3 pmol (LOD)	<a href="#">Roybal et al. (1989)</a>
Formulation	–	HPLC-PO-CL Column: C <sub>18</sub> Mobile phase: acetonitrile and 25 mM imidazole buffer containing 10 mM sodium 1-propanesulfonate pH 6.5 CL reaction solution: 0.25 mM TDPO and 25 mM H <sub>2</sub> O <sub>2</sub> in acetonitrile Flow rate: for eluent, 1 mL/min; and for CL solution, 1.3 mL/min	6 µg/mL (LOQ) 120 fmol (LOD)	<a href="#">Onur &amp; Acar (1992)</a> <a href="#">Kimoto et al. (1996)</a>

$\lambda_{ex}$ ,  $\lambda_{em}$ ,  $\lambda$  excitation;  $\lambda_{em}$ ,  $\lambda$  emission; CBA, carboxylic acid; CE-ESI-MS, capillary electrophoresis/electrospray ionization mass spectrometry; CE-UV, capillary electrophoresis ultraviolet spectroscopy; CL, chemiluminescence; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; FIA-PIF, flow injection analysis photochemically induced fluorescence; HPLC-PO-CL, high-performance liquid chromatography peroxyoxalate chemiluminescence; IEX-MS, ion exchange chromatography mass spectrometry; LC-ED, liquid chromatography electrochemical detection; LC-ESI-MS, liquid chromatography electrospray ionization mass spectrometry; LC-UV, liquid chromatography ultraviolet spectroscopy; LOD, limit of detection; LOQ, limit of quantitation; ppb, parts per billion; NaCl, sodium chloride; TDPO, bis(4-nitro-2-(3,6,9-trioxadecyloxy-carbonyl)phenyl)oxalate

### 1.3.2 Medical use

#### (a) Indications

Methylene blue is used in human and veterinary medicine for several therapeutic and diagnostic procedures, including as a stain in bacteriology, as a redox colorimetric agent, as a targeting agent for melanoma, as an antihaemoglobinaemic, as an antidote, and as an antiseptic and disinfectant (O'Neil *et al.*, 2006; NTP, 2008).

Methylene blue is used clinically in a wide range of indications, including the emergency treatment of methaemoglobinemia, ifosfamid-induced encephalopathy or poisoning by cyanide, nitrate or carbon monoxide, and for intraoperative tissue staining (Oz *et al.*, 2011; Schirmer *et al.*, 2011).

One of the most common clinical applications of methylene blue is for the treatment of methaemoglobinaemia induced by overexposure to drugs, to industrial chemicals such as nitrophenols (ATSDR, 1992), or to environmental poisons such as excessive nitrate in well-water, or cyanide compounds (Sills & Zinkham, 1994; Christensen *et al.*, 1996).

Methylene blue is used in the treatment of some psychiatric disorders because of the anxiolytic and antidepressant properties attributed to its ability to block activation of guanyl cyclase by nitric oxide (Naylor *et al.*, 1986; Eroğlu & Çağlayan, 1997). In 2011, however, the Food and Drug Administration of the United States issued a safety warning concerning the risk of serotonin syndrome when methylene blue is given concurrently with serotonergic psychiatric medications (FDA, 2011).

Recent studies suggested that methylene blue may have beneficial effects in the treatment of Alzheimer disease and memory improvement (Oz *et al.*, 2011).

The use of methylene blue as a candidate antimalarial drug was revived in 1995, with the major goal to develop an affordable, available, and accessible therapy for uncomplicated falciparum

malaria in children in Africa. In malaria combination therapy, methylene blue is also advantageous because the blue colour of the urine can be used as an indicator that the drug combination containing methylene blue has not been counterfeited, which is a serious problem in developing countries (Schirmer *et al.*, 2011). Some phase II trials have shown promising results, especially when methylene blue is combined with a more rapidly acting partner drug (Zoungrana *et al.*, 2008; Coulibaly *et al.*, 2009; Bountogo *et al.*, 2010).

#### (b) Dosage

In clinical use, methylene blue is either dissolved in sterile water to a concentration of 10 mg/mL (1%) injectable solution or administered orally in gelatin capsules to avoid staining of the oral mucous membranes and to ensure complete gastrointestinal delivery (Oz *et al.*, 2011). The dosage depends on the therapeutic indication (Schirmer *et al.*, 2011). For inherited methaemoglobinaemia, the suggested oral dosage was  $1 \times 50$ –250 mg/day (for a lifetime), while for acute methaemoglobinaemia the suggested dosage was  $1$ – $2 \times 1.3$  mg/kg body weight (bw), given intravenously over 20 minutes. In ifosfamid-induced neurotoxicity, oral or intravenous doses of  $4 \times 50$  mg/day were used. For prevention of urinary-tract infections in elderly patients, a dose of  $3 \times 65$  mg/day was given orally. In Alzheimer disease, the dosage was  $3 \times 60$  mg/day, and for paediatric malaria it was  $2 \times 12$  mg/kg bw orally for 3 days (Schirmer *et al.*, 2011). In a controlled trial in semi-immune adults with uncomplicated falciparum malaria, the oral dosage was 390 mg twice per day (Bountogo *et al.*, 2010). According to Medscape (2013), a solution (10 mg/mL) may be injected at the following intravenous dosages:  $1$ – $2$  mg/kg bw over 5–10 minutes for methaemoglobinaemia, and 50 mg every 6 to 8 hours until symptoms resolve for prevention of ifosfamid-induced encephalopathy.

### (c) Sales volume

Worldwide sales of methylene blue totalled US\$ 44 million in 2012, with 59% occurring in the USA. The only other nation to report substantial sales volumes was Brazil (US\$ 11 million) ([IMS Health, 2012](#)).

#### 1.3.3 Other uses

Methylene blue is used as a disinfectant and biological stain ([NTP, 2008](#); [Oz et al., 2011](#)). As a disinfectant, methylene blue is sold to end-consumers as an aquarium fungicide ([Schirmer et al., 2011](#)). Most recently, methylene blue has been used as an optical probe in biophysical systems, as an intercalator in nanoporous materials, as a redox mediator, and in photoelectrochromic imaging ([NTP, 2008](#)).

Methylene blue is used to dye paper and office supplies, but also to tone up silk colours ([Berneth, 2008](#)). In analytical chemistry, methylene blue is applied to determine anionic surfactants, which are termed “methylene blue active substances” ([Kosswig, 2000](#)). Methylene blue is also used in pH and redox indicator reagents ([Sabnis et al., 2009](#)).

## 1.4 Occurrence and exposure

### 1.4.1 Natural occurrence

Methylene blue is a synthetic substance and does not occur naturally.

### 1.4.2 Occupational exposure

A National Occupational Exposure Survey in the USA indicated that an estimated 69 563 workers were potentially exposed to methylene blue in the workplace between 1981 and 1983 ([NTP, 2008](#)).

### 1.4.3 General population and consumers

In 20 paediatric patients in Burkina Faso who were treated for malaria with methylene blue at an oral dose of 20 mg/kg bw, the concentrations

in samples of dried whole blood on paper spots ranged between 531 and 2645 ng/mL within 1 hour after administration ([Burhenne et al., 2008](#)). In a phase 1 study of malaria treatment, mean plasma concentrations after a single dose of methylene blue in healthy adults were 748 ng/mL (50 mg, intravenous injection;  $n = 16$ ) and 3905 ng/mL (500 mg, oral administration;  $n = 16$ ) ([Walter-Sack et al., 2009](#)).

No systematic data on other exposures, e.g. environmental contamination, were available to the Working Group. While methylene blue may hypothetically enter the food chain after application in veterinary medicine (which would be illegal in most jurisdictions), or as a contaminant in drinking-water, no systematic data on residue levels in food or water were available. In the few available studies, it was found that metabolites rather than methylene blue itself were detectable, e.g. in milk from dairy cattle treated with methylene blue ([Roybal et al., 1996](#)).

## 1.5 Regulations and guidelines

No permissible exposure limits for methylene blue have been established in the USA by the Occupational Safety and Health Administration, the National Institute for Occupational Safety and Health, or the American Conference of Governmental Industrial Hygienists ([NTP, 2008](#)). In the European Union, the use of methylene blue in food-producing animals is not allowed. According to [Xu et al. \(2009\)](#), Japan has established a maximum residue limit of 10 µg/kg for methylene blue in aquatic products, because it is used as a replacement for other antifungal dyes in aquaculture.

Specifications for methylene blue are published in several official pharmacopoeias ([Table 1.2](#)).



**Table 1.2 Specifications for methylene blue**

Parameter	WHO International Pharmacopoeia, 4th edition	United States Pharmacopoeia 36	European Pharmacopoeia 7.0
Content C <sub>16</sub> H <sub>18</sub> ClN <sub>3</sub> S (dried substance)	97.0–101.0%	98.0–103.0%	95.0–101.0%
Identity tests	A. IR B. Colour reaction with hydrochloric acid and zinc powder C. General identification test as characteristic of chlorides	IR	A. UV/VIS B. TLC C. Colour reaction with glacial acetic acid and zinc powder D. Reaction of chlorides
Copper or zinc	Absence of zinc; copper, max. 0.20 mg/g	Absence of zinc; copper max. 0.02%	Zinc, max. 100 ppm; copper, max. 300 ppm
Metals besides copper and zinc	Iron, max. 0.10 mg/g	Arsenic, max. 8 ppm	Max. contents: aluminium, 300 ppm; cadmium, 1 ppm; chromium, 100 ppm; tin, 10 ppm; iron, 200 ppm; manganese, 10 ppm; mercury, 1 ppm; molybdenum, 10 ppm; nickel, 10 ppm; lead, 10 ppm
Sulfated ash	Max. 10 mg/g		Max. 0.25%
Loss on drying	80–220 mg/g	8.0–18.0%	8.0–22.0%
Foreign substances/chromatographic purity/related substances	TLC: no spots besides the characteristic spots	TLC: max. four spots	HPLC: detailed specification of max. peak areas of impurities
Residue on ignition		Max. 1.2%	
Organic volatile impurities		Meets the requirements	
Bacterial endotoxins	Max. 2.5 IU of endotoxin per mg		
Methanol-insoluble substances			Max. 10.0 mg (1.0%)

HPLC, high-performance liquid chromatography; IR, infrared; IU, international unit; max., maximum; TLC, thin-layer chromatography; UV/VIS, ultraviolet and visible absorption spectrophotometry

From [EDQM \(2008\)](#), [WHO \(2011\)](#), [US Pharmacopoeial Convention \(2013\)](#)

## 2. Cancer in Humans

No data were available to the Working Group.

## 3. Cancer in Experimental Animals

### 3.1 Mouse

In a study of oral administration, groups of 50 male and female B6C3F<sub>1</sub> (age, 6 weeks) received methylene blue (in a 0.5% aqueous methylcellulose solution) at a dose of 0 (control), 2.5, 12.5, or 25 mg/kg bw per day by gavage on 5 days per week for up to 106 weeks. There was an increase in mean body weight in females at the intermediate and highest doses compared with controls. Survival of treated groups was similar to that of controls.

In males, there was a significant positive increase in the trend in the incidence of carcinoma ( $P=0.027$ , poly-3 trend test) and of adenoma or carcinoma (combined) of the small intestine ( $P = 0.029$ , poly-3 trend test). The incidences of carcinoma were: 0/50 (0%), 1/50 (2%), 2/50 (4%), 4/50 (8%); and the incidences of adenoma or carcinoma (combined) were: 1/50 (2%), 2/50 (4%), 4/50 (8%), 6/50 (12%). The incidences in the dosed groups were not significant by pairwise comparison. The incidence of adenoma or carcinoma (combined) in the group receiving the highest dose (12%) exceeded the range for historical controls (39/1508; range, 0–10%); while the incidence in controls (2%) was consistent with the range for historical controls.

In males, the incidence of bronchiolo-alveolar carcinoma of the lung occurred with a significant positive trend: 1/50 (2%), 4/50 (8%), 5/50 (10%), 7/50 (14%);  $P = 0.043$ , poly-3 trend test); and the incidence was significantly increased in the group at the highest dose ( $P = 0.039$ ; poly-3 test). The incidence in males receiving methylene blue were within the range for historical controls

for all routes of administration (151/1507; range, 4–24%) and the incidence in controls in the current study was below the range for historical controls. [The Working Group considered that the significantly increased incidence and significant positive trend in the incidence of bronchiolo-alveolar carcinoma was therefore not related to treatment with methylene blue.] In females, the incidences of bronchiolo-alveolar carcinoma were decreased in all groups of treated mice (5/50, 0/50, 0/50, 1/50), and the decreases were significant ( $P \leq 0.05$ , poly-3 test) in the groups receiving the lowest and intermediate dose.

The incidence of malignant lymphoma in females occurred with a significant positive trend: 6/50 (12%), 4/50 (8%), 9/50 (18%), 12/50 (24%);  $P = 0.025$ , poly-3 trend test. However, the incidence in females at the highest dose (24%) was well within the range for historical controls (308/1508; range, 6–58%) for this neoplasm with a highly variable incidence. In males, the incidences were 2/50 (4%), 2/50 (4%), 2/50 (4%), 5/50 (10%). While the incidence in the group at the highest dose was higher than in controls, it was not significantly increased, and barely exceeded the range for historical controls (70/1508; range, 0–8%) ([NTP, 2008](#); [Auerbach et al., 2010](#)).

### 3.2 Rat

In a study of oral administration, groups of 50 male and 50 female F344/N rats (age, 6 weeks) received methylene blue in a 0.5% aqueous methylcellulose solution at a dose of 0 (control), 5, 25, or 50 mg/kg bw, by gavage once per day on 5 days per week for up to 106 weeks. The mean body weights of males and females in groups at the intermediate and highest dose were decreased compared with controls at the end of the study. There was no effect on body weight in groups at the lowest dose. Survival of treated groups was similar to that of the controls.

In males, the trend in the incidence of pancreatic islet cell adenoma and of adenoma

or carcinoma (combined) were non-significantly increased. The incidences of adenoma were: 4/50 (8%), 9/50 (18%), 12/50 (24%), and 8/50 (16%); and the incidences of adenoma or carcinoma (combined) were: 4/50 (8%), 9/50 (18%), 14/50 (28%), and 8/50 (16%). The incidences were significantly increased only in the group receiving the intermediate dose (adenoma,  $P = 0.037$ ; adenoma or carcinoma (combined),  $P = 0.013$ ; poly 3-test), and the incidence of islet cell carcinoma of the pancreas (2/50; 4%) in the group receiving the intermediate dose was within the range for historical controls (26/1448; range, 0–8%). [Although the incidence of pancreatic islet cell hyperplasia was significantly increased in the group at the highest dose versus controls (26/50 versus 13/50;  $P \leq 0.01$ ), and in view of the fact that islet cell hyperplasia, adenoma, and carcinoma are thought to constitute a morphological and biological continuum in the progression of islet cell proliferation, the Working Group considered that the positive trend in the incidence of adenoma or carcinoma (combined) was mainly the result of the increased trend in the incidence of adenoma].

There was no increase in the incidence of any neoplasm in exposed females (NTP, 2008; Auerbach *et al.*, 2010).

## 4. Mechanistic and Other Relevant Data

### 4.1 Absorption, distribution, metabolism, and excretion

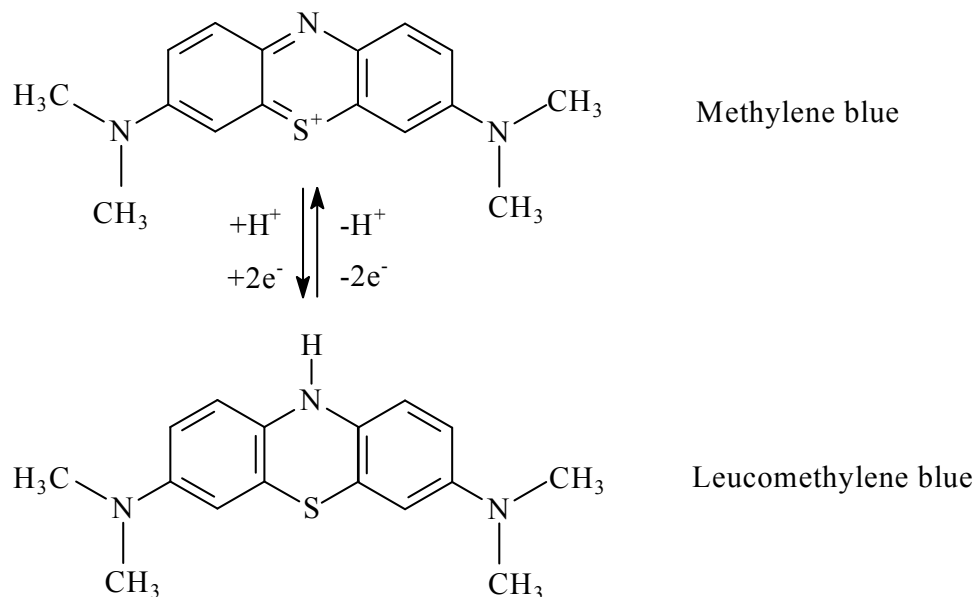
#### 4.1.1 Humans

After an intravenous bolus injection of 100 mg, the mean plasma concentration of methylene blue was reported to be 5  $\mu\text{M}$  in healthy volunteers [number not specified] (Aeschlimann *et al.*, 1996).

Methylene blue is well absorbed, reduced, and excreted largely in the urine as the reduced leucomethylene blue (colourless) form (DiSanto & Wagner, 1972a; Fig. 4.1). The *N*-demethylated metabolites azure A (minor), azure B, and azure C (minor), which have the potential to undergo deprotonation to a neutral quinone imine, have been reported (Munns *et al.*, 1992; Schirmer *et al.*, 2011; Fig. 4.2), but their pharmacokinetic characteristics do not appear to have been investigated. One study mentioned the presence of azure B in autopsied peripheral organs from a patient who had received 200 mg of methylene blue intravenously, at levels (475–2943 ng/g) higher than those (74–208 ng/g) of methylene blue in the same tissues (Warth *et al.*, 2009). [The Working Group noted that the metabolites of methylene blue are anticipated to have greater lipophilicity than the parent compound and may accumulate in tissues.]

When administered orally to seven healthy human subjects at a dose of 10 mg in capsule form, the total urinary recovery ranged from 53% to 97% of the administered dose, with an average of 74%. Of the material recovered, an average of 78% was excreted as leucomethylene blue and the remainder as methylene blue. Excretion rate–time plots for methylene blue and leucomethylene blue suggested a circadian rhythm (DiSanto & Wagner, 1972a).

In another study, the concentration of methylene blue in whole blood was measured in healthy individuals, before and after oxidation, following intravenous ( $n = 7$ ) or oral ( $n = 7$ ) administration of 100 mg of methylene blue. The concentration of methylene blue in whole blood after intravenous administration showed a multiphasic time course with an estimated terminal half-life of 5.25 hours. The area under the curve (AUC) was  $0.134 \pm 0.025 \mu\text{mol/mL}\cdot\text{min}$  and the systemic clearance was  $3.0 \pm 0.7 \text{ L/min}$ . After oral administration (in capsule form), maximum concentrations were reached within 1–2 hours; the AUC ( $0.01 \pm 0.004 \mu\text{mol/mL}\cdot\text{min}$ )

**Fig. 4.1 Structures of methylene blue and leucomethylene blue**

Compiled by the Working Group

was one order of magnitude lower than upon intravenous administration. The urinary excretion of total methylene blue (methylene blue and leucomethylene blue), between 4 and 14 hours, was significantly ( $P < 0.01$ ) higher after intravenous administration than after oral administration ( $28.6 \pm 3.0\%$  and  $18.4 \pm 2.4\%$  of the administered dose, respectively). In this study, approximately one third of the methylene blue excreted in the urine was in the leuco form (Peter *et al.*, 2000).

Another study compared the administration of single doses of methylene blue: 50 mg intravenously ( $n = 16$ ) versus 500 mg orally ( $n = 16$ ). The mean plasma AUCs were estimated to be  $7.6 \pm 3.4 \mu\text{g/mL}\cdot\text{h}$  and  $51.2 \pm 17.1 \mu\text{g/mL}\cdot\text{h}$  after intravenous and oral administration, respectively. The absolute bioavailability was  $72.3 \pm 23.9\%$  (Walter-Sack *et al.*, 2009).

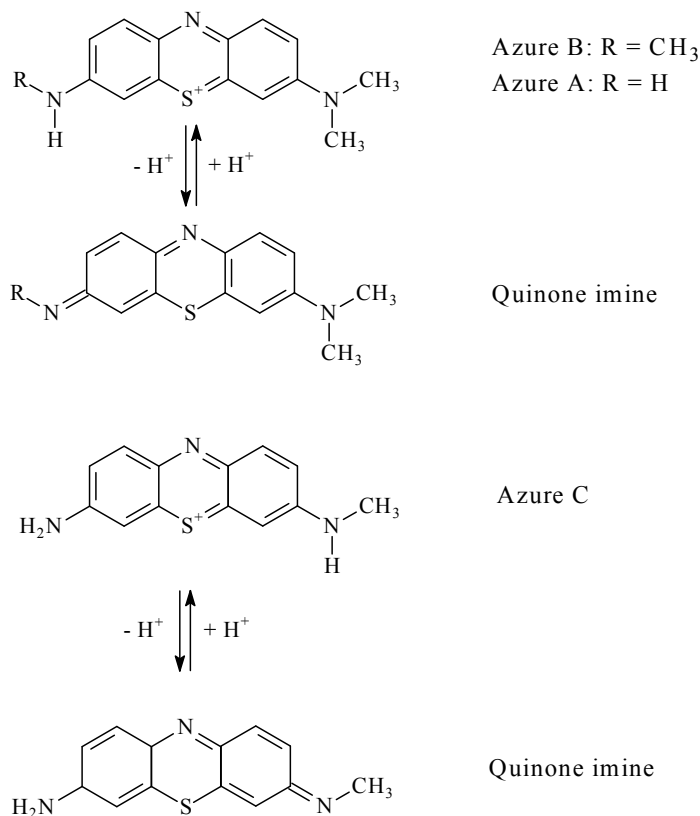
The pharmacokinetics of methylene blue were investigated in the setting of lymphatic mapping of cancer of the breast. A subareolar injection of 4 mL of a methylene blue solution at 1.25 mg/mL (total dose, 5 mg) resulted in rapid absorption

(time to peak, 23 minutes) and an average peak serum concentration of 71.3 ng/mL. The elimination was slow ( $t_{1/2} = 11.1$  hours), and 32% of the initial dose was recovered within 48 hours. The highest serum concentration was 280 ng/mL (Pruthi *et al.*, 2011). Of note, methylene blue concentrations have been found to be four- to fivefold higher in whole blood than in plasma (Peter *et al.*, 2000; Rengelshausen *et al.*, 2004).

[The Working Group noted that leucomethylene blue is readily oxidized in air and forms stable complexes in the urine, but not blood (DiSanto & Wagner, 1972b, c). It is not clear whether or not discrepancies in the relative proportions of methylene blue and the leuco form between studies may be due to different aeration conditions during sample processing.]

#### 4.1.2 Experimental animals

In one male and one female dog given methylene blue orally at a dose of 15 mg/kg bw, methylene blue was not detectable in the blood. The female was catheterized and urine was collected

**Fig. 4.2 Structures of the methylene blue metabolites azure B, azure A, and azure C**

Compiled by the Working Group

for 10 hours after dosing; the recovery was 2.4% of the administered dose. When the female was given methylene blue orally at a dose of 10 mg/kg bw, 3.8% of the administered dose was recovered in the urine within 14 hours (DiSanto & Wagner, 1972a). In comparison with the data obtained for humans in the same study (see Section 4.1.1), this low recovery indicated that methylene blue is well absorbed in humans and poorly absorbed in dogs after oral administration.

In another study, male Sprague-Dawley rats were treated intravenously with methylene blue at a dose of 2–25 mg/kg bw and killed 3 minutes after dosing; lungs, liver, kidneys, and heart were removed and assayed for methylene blue. An average of 29.8% of the administered dose (range, 25.2–35.8%) was recovered in the four tissues, which is consistent with very rapid and

extensive uptake of methylene blue by tissues; the uptake was best described by a nonlinear model (DiSanto & Wagner, 1972c).

The distribution of total methylene blue in different tissues of male Wistar rats was measured after intravenous or intraduodenal administration of a single dose at 10 mg/kg bw. The rats were killed after 1 hour and samples from several different tissues were collected. The concentrations of the drug in the blood and brain were significantly higher ( $P < 0.05$ ) after intravenous than after intraduodenal administration. In contrast, the concentrations in the intestinal wall and in the liver were significantly ( $P < 0.05$ ) higher after intraduodenal administration, while concentrations in bile, and biliary excretion, were not affected by the route of administration. Less than 3% of the administered dose was found in

the intestinal lumen 1 hour after intraduodenal administration ([Peter et al., 2000](#)).

When a 10% solution of methylene blue was administered by intramammary infusion to lactating goats, the drug passed quickly into systemic circulation, peaked at 3 hours, and was still detectable in the blood 12 hours after infusion ([Ziv & Heavner, 1984](#)).

Azure B, together with methylene blue and leucomethylene blue, was reported to be present in the urine of male and female Fischer 344 rats ( $n = 5$ ) given methylene blue as a single intravenous dose of 2.5 mg/kg bw, or a single oral dose of either 2.5 or 50 mg/kg bw. The methylene blue used in the experiment was contaminated with azure B at approximately 15%; metabolism of methylene blue through *N*-demethylation was inferred from a time-dependent increase in the amount of azure B present in the urine, but quantification of azure B was not provided ([Gaudette & Lodge, 2005](#)).

Methylene blue was reported to bind strongly to rabbit plasma (71–77% of bound drug). Extensive tissue and protein binding was proposed to account for the high apparent volume of distribution (21 L/kg) in rabbits ([Kozaki & Watanabe, 1981](#)).

## 4.2 Genetic and related effects

See [Table 4.1](#)

### 4.2.1 Humans

In mucosal cells from Barrett oesophagus in humans undergoing endoscopy, methylene blue dye (0.5% solution) (which was used to identify specific areas of interest for biopsy) induced DNA damage as detected by the alkaline comet assay and the modified comet assay using the enzyme formamide pyrimidine-DNA glycosylase (FPG) to detect damage associated with reactive oxygen species ([Olliver et al., 2003](#)). Fifteen patients undergoing endoscopy were biopsied at

oesophageal mucosal sites that were treated with methylene blue, and at adjacent sites not treated with methylene blue. Comet assays revealed that elevated levels of DNA damage were observed in oesophageal mucosal cells exposed to methylene blue in all 15 patients, while samples adjacent to the methylene blue-exposed sites had significantly lower levels of DNA damage, despite photosensitization with white light from the endoscope ([Olliver et al., 2003](#)). Exposure in vitro of normal oesophageal tissue, obtained by biopsy, to methylene blue (0.5% for 1 minute) in the absence of light did not result in an increase in DNA damage ([Olliver et al., 2003](#)), confirming the role of white light-activated methylene blue in the induction of DNA damage. Similarly, an increase in DNA damage (alkali-labile sites and FPG-sensitive sites) was seen in biopsied colonic epithelium sprayed with methylene blue dye (0.1%) during colonoscopy (which used illumination with white light) compared with colonic epithelial cells sampled in the same region before spraying with methylene blue ([Davies et al., 2007](#)).

### 4.2.2 Experimental systems

#### (a) Mutation

##### (i) Assays in bacteria or yeast

Methylene blue was shown to be mutagenic without photoactivation in a variety of *Salmonella typhimurium* tester strains, inducing both base-substitution and frameshift mutations, with and without metabolic activation ([Chung et al., 1981](#); [Yamaguchi, 1981](#); [Lunn & Sansone, 1991](#); [NTP, 2008](#)); mutagenic activity or induction of DNA damage was also reported in several strains of *Escherichia coli* ([McCarroll et al., 1981](#); [Mohn et al., 1984](#); [Webb & Hass, 1984](#); [NTP, 2008](#)). In contrast, photoactivated (664 nm) methylene blue did not induce gene conversion in the yeast *Saccharomyces cerevisiae* ([Ito & Kobayashi, 1977](#)), and no induction of gene mutation was seen in *S. cerevisiae* treated

**Table 4.1 Genetic and related effects of methylene blue and its metabolites**

Test system	Results <sup>a</sup>		Dose (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system <sup>b</sup>		
<i>Methylene blue</i>				
Bacteriophage PM2 cell-free, DNA damage, in the presence of white-light activation	+	NT	10 µg/mL	<a href="#">Epe et al. (1988)</a>
Bacteriophage pAQ1 in <i>Salmonella typhimurium</i> TA1535 and TA1978, DNA damage, in the presence of white-light activation	+ <sup>c</sup>	NT	10 µM	<a href="#">Epe et al. (1989)</a>
Bacteriophage PM2 cell-free, DNA damage, in the presence of white-light activation	+ <sup>c</sup>	NT	27 µM	<a href="#">Epe et al. (1993)</a>
Bacteriophage pAQ1 in <i>Salmonella typhimurium</i> TA1978, DNA damage in PM2 with white-light activation	+ <sup>c</sup>	NT	27 µM	<a href="#">Epe et al. (1993)</a>
Single-stranded M13mp2 bacteriophage, DNA damage with photoactivation <sup>d</sup>	+	NT	2.5 µM	<a href="#">McBride et al. (1992)</a>
Calf thymus DNA, intercalation, with photoactivation	+	NT	1.83 µM	<a href="#">Lee et al. (1973)</a>
Calf thymus DNA, intercalation, with photoactivation	+	NT	NR <sup>e</sup>	<a href="#">Nordén &amp; Tjerneld (1982)</a>
DNA-protein crosslinks, calf thymus DNA, calf thymus histone type II, with photoactivation	+	NT	5 µM	<a href="#">Villanueva et al. (1993)</a>
<i>Salmonella typhimurium</i> TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	+ (TA98)	+ (TA98)	5 µg/plate	<a href="#">Chung et al. (1981)</a>
<i>Salmonella typhimurium</i> TA100, reverse mutation	+	+	20 µg/plate	<a href="#">Yamaguchi (1981)</a>
<i>Salmonella typhimurium</i> TA100, TA1530, TA1535, TA98, reverse mutation	+ (TA1530, TA98)	+ (TA98)	1000 µg/plate	<a href="#">Lunn &amp; Sansone (1991)</a>
<i>Salmonella typhimurium</i> TA100, reverse mutation	(±)	+ <sup>f</sup>	33 µg/plate	<a href="#">NTP (2008)</a>
<i>Salmonella typhimurium</i> TA98, reverse mutation	+	+ <sup>f</sup>	33 µg/plate, -S9 3.3 µg/plate, +S9	<a href="#">NTP (2008)</a>
<i>Salmonella typhimurium</i> TA100, reverse mutation	+	+	0.25 µg/plate, -S9 10 µg/plate, +S9	<a href="#">NTP (2008)</a>
<i>Salmonella typhimurium</i> TA98, reverse mutation	+	+	1 µg/plate, -S9 10 µg/plate, +S9	<a href="#">NTP (2008)</a>
<i>Salmonella typhimurium</i> TA1535, TA1538, reverse mutation, with and without photoactivation	+ (TA1535) <sup>g</sup>	NT	20 µg/plate	<a href="#">Gutter et al. (1977)</a>
<i>Salmonella typhimurium</i> TA1535, TA2638, TA100, TA104, reverse mutation, with photoactivation	+	NT	10 µg/mL	<a href="#">Epe et al. (1989)</a>

Table 4.1 (continued)

Test system	Results <sup>a</sup>		Dose (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system <sup>b</sup>		
<i>Escherichia coli</i> WP2, WP2 <i>uvrA</i> -, WP67 <i>uvrA</i> -, WP611 <i>uvrA</i> - <i>lexA</i> -, WP100 <i>uvrA</i> - <i>recA</i> -, W3110 <i>polA</i> <sup>+</sup> , p3478 <i>polA</i> -, DNA damage	+ (CM611, WPI00, p3478)	NT	160 µg/well (p3478 <i>polA</i> -)	<a href="#">McCarroll et al. (1981)</a>
<i>Escherichia coli</i> AB1157, B/r, WP2, WP2s, WP10, WP6 ( <i>polA1</i> ), resistance to bacteriophage T5	+ (AB1157, WP2s, WP10) WP10	NT	2 µM	<a href="#">Webb &amp; Hass (1984)</a>
<i>Escherichia coli</i> K-12/343/113, reverse mutation to Arg <sup>+</sup> , with white-light activation	+	NT	10–40 µM (LED, NR)	<a href="#">Mohn et al. (1984)</a>
<i>Escherichia coli</i> WP2 <i>uvrA</i> pKM101, reverse mutation	+	+	0.5 µg/plate, -S9 25 µg/plate, +S9	<a href="#">NTP (2008)</a>
<i>Saccharomyces cerevisiae</i> , gene conversion, with white light photoactivation (λ <sub>max</sub> 664 nm)	-	NT	0.95 (OD <sub>λ<sub>max</sub></sub> ) <sup>b</sup>	<a href="#">Ito &amp; Kobayashi (1977)</a>
<i>Saccharomyces cerevisiae</i> 507.4/2b, MT182/8d, CM106/5a, gene mutations, no photoactivation	-	NT	20 µg/mL	<a href="#">Tuite et al. (1981)</a>
Bacteriophage <i>Serratia</i> phage <i>kappa</i> , mutagenicity, with photoactivation	+	NT	NR	<a href="#">Brendel (1973)</a>
DNA damage (alkali-labile sites) (comet assay), male Sprague-Dawley rat, primary hepatocytes, with visible light activation in vitro	+	NT	0.31 µM × 2 min	<a href="#">Lábaj et al. (2007)</a>
DNA damage (FPG-sensitive sites) (comet assay), male Sprague-Dawley rat, primary hepatocytes, with visible light activation in vitro	+	NT	0.31 µM × 2 min	<a href="#">Lábaj et al. (2007)</a>
DNA damage (alkali-labile sites; FPG-sensitive sites) (comet assay), male Sprague Dawley rat, primary hepatocytes, in vitro	-	NT	0.31 µM × 3 min	<a href="#">Lábaj et al. (2007)</a>
DNA damage (alkali-labile sites; FPG-sensitive sites) (comet assay), male Sprague-Dawley rat, primary hepatocytes, in vitro	+	NT	0.31 µM × 3 min	<a href="#">Horváthová et al. (2012)</a>
DNA damage (alkali-labile sites) (comet assay), male Sprague Dawley rat, primary hepatocytes, with visible light activation in vitro	+	NT	0.31 µM × 3 min	<a href="#">Horváthová et al. (2012)</a>
DNA damage (FPG-sensitive sites) (comet assay), male Sprague-Dawley rat, primary hepatocytes, with visible light activation in vitro	+	NT	0.31 µM × 3 min	<a href="#">Horváthová et al. (2012)</a>
DNA damage (alkali-labile sites) (comet assay), MCF-7 cells, with visible light activation in vitro	+	NT	0.1% × 5 min	<a href="#">Masannat et al. (2009)</a>
DNA damage (FPG-sensitive sites) (comet assay), MCF-7 cells, with visible light activation in vitro	-	NT	1.0% × 5 min	<a href="#">Masannat et al. (2009)</a>
DNA damage (alkali-labile sites) (comet assay), HB-2 cells, with visible light activation in vitro	+	NT	1.0% × 5 min	<a href="#">Masannat et al. (2009)</a>
DNA damage (FPG-sensitive sites) (comet assay), HB-2 cells, with visible light activation in vitro	-	NT	1.0% × 5 min	<a href="#">Masannat et al. (2009)</a>
DNA damage (comet assay), CaCo-2 cells, in vitro	-	NT	0.1% × 2 min	<a href="#">Davies et al. (2007)</a>



Table 4.1 (continued)

Test system	Results <sup>a</sup>		Dose (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system <sup>b</sup>		
DNA damage (alkali-labile sites) (comet assay), CaCo-2 cells, with visible light activation in vitro	+	NT	0.1% × 2 min	<a href="#">Davies et al. (2007)</a>
DNA damage (FPG-sensitive sites) (comet assay), CaCo-2 cells, with visible light activation in vitro	+	NT	0.1% × 2 min	<a href="#">Davies et al. (2007)</a>
DNA damage (alkali-labile sites) (comet assay), human colonic mucosa cells, with visible light activation during colonoscopy in vivo	+		0.1%	<a href="#">Davies et al. (2007)</a>
DNA damage (FPG-sensitive sites) (comet assay), human colonic mucosa cells, with visible light activation during colonoscopy in vivo	+		0.1%	<a href="#">Davies et al. (2007)</a>
DNA damage (comet assay), human Barrett oesophagus cells (biopsy), in vitro	-	NT	0.5% × 1 min	<a href="#">Olliver et al. (2003)</a>
DNA damage (alkali-labile sites) (comet assay), human Barrett oesophagus cells, with visible light activation during endoscopy in vivo	+		0.5%	<a href="#">Olliver et al. (2003)</a>
DNA damage (FPG-sensitive sites) (comet assay), human Barrett oesophagus cells, with visible light activation during endoscopy in vivo	+		0.5%	<a href="#">Olliver et al. (2003)</a>
DNA damage (alkali-labile sites) (comet assay), human OE33 cells, with white-light activation in vitro	+	NT	15 mM (0.5%) × 5 min	<a href="#">Sturmey et al. (2009)</a>
DNA damage (alkali-labile sites) (comet assay), human OE33 cells, with red light activation in vitro	+	NT	15 mM (0.5%) × 5 min	<a href="#">Sturmey et al. (2009)</a>
DNA damage (FPG-sensitive sites) (comet assay), human OE33 cells, with red light activation in vitro	+	NT	1.5 mM × 5 min	<a href="#">Sturmey et al. (2009)</a>
DNA damage (alkali-labile sites) (comet assay), human OE33 cells, with green light activation in vitro	-	NT	15 mM (0.5%) × 3 min	<a href="#">Sturmey et al. (2009)</a>
DNA damage (alkali-labile sites) (comet assay), human OE33 cells, with blue light activation in vitro	-	NT	15 mM (0.5%) × 3 min	<a href="#">Sturmey et al. (2009)</a>
DNA damage (alkali-labile sites) (comet assay), human OE33 cells, with filtered white light (to remove 580–800 nm red spectrum) activation in vitro	-	NT	15 mM (0.5%) × 3 min	<a href="#">Sturmey et al. (2009)</a>
<i>Drosophila melanogaster</i> , sex-linked recessive lethal mutation, in germ cells, larval feeding	-		0.1% in feed	<a href="#">Clark (1953)</a>
<i>Drosophila melanogaster</i> , somatic mutation and recombination test (SMART), with photoactivation	+		0.01 mM in feed	<a href="#">Smijs et al. (2004)</a>
Sister-chromatid exchange, Chinese hamster V79 cells, in vitro	-	NT	1.0 µg/mL	<a href="#">Popescu et al. (1977)</a>

**Table 4.1 (continued)**

Test system	Results <sup>a</sup>	Dose (LED or HID)	Reference
Sister-chromatid exchange, Chinese hamster V79 cells, in vitro, no photoactivation	+	0.1 µg/mL	<a href="#">Speit &amp; Vogel (1979)</a>
Sister-chromatid exchange, Chinese hamster V79 cells, in vitro, with photoactivation	-	1.0 µg/mL	<a href="#">Speit &amp; Vogel (1979)</a>
Sister-chromatid exchange, Syrian hamster BHK-1 cells, with/without photoactivation in vitro	-	27 µg/mL	<a href="#">MacRae et al. (1980)</a>
Sister-chromatid exchange, Chinese hamster ovary cells, in vitro	+	0.63 µg/mL (-S9) 4.7 µg/mL (+S9)	<a href="#">NTP (2008)</a>
Chromosomal aberrations, Chinese hamster ovary cells, in vitro	-	20 µM <sup>i</sup>	<a href="#">Au &amp; Hsu (1979)</a>
Chromosomal aberrations, Chinese hamster V79 cells, in vitro	-	1.0 µg/mL	<a href="#">Popescu et al. (1977)</a>
Chromosomal aberrations, Chinese hamster ovary cells, in vitro	+	7.5 µg/mL (-S9) 4.7 µg/mL (+S9)	<a href="#">NTP (2008)</a>
Sister chromatid exchanges, Chinese hamster bone-marrow cells, in vivo	-	12 mg/kg bw, ip × 1	<a href="#">Speit (1982)</a>
Micronucleus formation, male B6C3F <sub>1</sub> mice, bone-marrow cells or peripheral blood erythrocytes, in vivo	-	150 mg/kg bw, ip × 1	<a href="#">NTP (2008)</a>
Micronucleus formation, male and female B6C3F <sub>1</sub> mice, peripheral blood erythrocytes, in vivo	-	200 mg/kg bw per day, gavage × 14 wk	<a href="#">NTP (2008)</a>
<i>Azure A</i>			
<i>Salmonella typhimurium</i> TA100, reverse mutation	+	10 µg/plate, -S9 50 µg/plate, +S9	<a href="#">NTP (2008)</a>
<i>Salmonella typhimurium</i> TA98, reverse mutation	+	10 µg/plate, -S9 100 µg/plate, +S9	<a href="#">NTP (2008)</a>
<i>Escherichia coli</i> WP2 <i>uvrA</i> pKM101, reverse mutation	+	50 µg/plate, -S9 250 µg/plate, +S9	<a href="#">NTP (2008)</a>
Chromosomal aberrations, Chinese hamster ovary cells, in vitro	+	10 µM <sup>i</sup>	<a href="#">Au &amp; Hsu (1979)</a>
<i>Azure B</i>			
<i>Salmonella typhimurium</i> TA100, TA98, reverse mutation	+	10 µg/plate	<a href="#">NTP (2008)</a>
<i>Escherichia coli</i> WP2 <i>uvrA</i> pKM101, reverse mutation	+	10 µg/plate, -S9 100 µg/plate, +S9	<a href="#">NTP (2008)</a>
Chromosomal aberrations, Chinese hamster ovary cells, in vitro	+	20 µM <sup>i</sup>	<a href="#">Au &amp; Hsu (1979)</a>

Table 4.1 (continued)

Test system	Results <sup>a</sup>		Dose (LED or HID)	Reference
	Without exogenous metabolic system <sup>b</sup>	With exogenous metabolic system <sup>b</sup>		
<i>Azure C</i>				
<i>Salmonella typhimurium</i> TA100, reverse mutation	+	+	25 µg/plate, -S9 100 µg/plate, +S9	<a href="#">NTP (2008)</a>
<i>Salmonella typhimurium</i> TA98, reverse mutation	+	+	10 µg/plate, -S9 250 µg/plate, +S9	<a href="#">NTP (2008)</a>
<i>Escherichia coli</i> WP2 <i>uvrA</i> pKM101, reverse mutation	+	+	25 µg/plate, -S9 100 µg/plate, +S9	<a href="#">NTP (2008)</a>
Chromosomal aberrations, Chinese hamster ovary cells, in vitro	+	NT	20 µM <sup>f</sup>	<a href="#">Au &amp; Hsu (1979)</a>

<sup>a</sup> +, positive; -, negative; (±), equivocal

<sup>b</sup> S9 from Aroclor 1254-treated Sprague-Dawley rats, unless otherwise noted

<sup>c</sup> DNA damage was in the form of base modifications consistent with singlet oxygen generation

<sup>d</sup> 8-hydroxydeoxyguanosine and SOS-induced mutations implicating generation of lesions (ionic) other than 8-hydroxydeoxyguanosine in methylene blue plus white light oxidative DNA damage

<sup>e</sup> Intercalation orientation is changed by ionic strength; at low ionic strength, methylene blue is oriented co-planar with the DNA bases and at higher ionic strength, orientation changes

<sup>f</sup> S9 from Aroclor 1254-treated Sprague-Dawley rats or Syrian hamsters

<sup>g</sup> Photoactivation required; no increase in mutations in the absence of photoactivation with white light. Dose-response observed in the presence of white light (2-hour exposure) over a

range of 10–100 µg/plate

<sup>h</sup> Concentrated stock solution was diluted with 0.067 M phosphate buffer to give a final concentration of OD ≥ 1 at its absorption peak

<sup>i</sup> Not possible to accurately interpret the data; duration of exposure was only 5 hours, only 50 cells were evaluated for aberrations per concentration tested, gaps were included in the overall assessment of chromosomal damage, and data were presented as total aberrations rather than percentage of aberrant cells

<sup>j</sup> Not possible to accurately interpret the data; high levels of cytotoxicity were noted at ≥ 10 µM for azure A. For azure B and C, only the cytotoxic concentration (20 µM) was tested

bw, body weight; HID, highest ineffective dose; ip, intraperitoneal; LED, lowest effective dose; min, minute; NR, not reported; NT, not tested; po, oral; wk, week

with methylene blue at a single concentration of 20 µg/mL in the absence of photoactivation (Tuite *et al.*, 1981). It was suggested that the negative results in the yeast assays resulted from the inability of methylene blue to penetrate the yeast cell wall (Ito & Kobayashi, 1977).

(ii) *Drosophila melanogaster*

No increase in the frequency of sex-linked recessive lethal mutation was detected in germ cells of male *Drosophila melanogaster* given methylene blue via a larval feeding regimen (Clark, 1953). However, when photoactivated with white light, methylene blue induced high levels of homologous mitotic recombination in a somatic mutation and recombination test (SMART) in *D. melanogaster* (Smijs *et al.*, 2004).

(b) DNA damage

Positive results were reported in several in-vitro tests for mutagenicity or DNA damage induction with photoactivated methylene blue, presumably the result of singlet oxygen production (Brendel, 1973; Gutter *et al.*, 1977; Epe *et al.*, 1988, 1989, 1993; McBride *et al.*, 1992).

Methylene blue was shown to intercalate into calf thymus DNA (Lee *et al.*, 1973), and to bind to calf thymus DNA in an orientation perpendicular to the helix axis, coplanar with the bases, at low methylene blue : DNA binding ratios and low ionic strengths (Nordén & Tjerneld, 1982). Villanueva *et al.* (1993) reported that methylene blue induced light-dose-dependent increases in DNA–protein crosslinks (calf thymus DNA, calf thymus histone type II), which was attributed to the production of singlet oxygen.

Several studies of DNA damage using the comet assay have been conducted with the majority demonstrating a requirement for methylene blue activation by visible (white) light to induce both alkali-labile and FPG-sensitive (oxidized guanine) sites. Studies were conducted in male Sprague-Dawley rat primary hepatocytes (Lábaj *et al.*, 2007; Horváthová *et al.*, 2012), MCF-7 breast cancer cells (Masannat *et al.*, 2009),

HB-2 normal human breast cells (Masannat *et al.*, 2009), cultured colonic adenocarcinoma CaCo-2 cells (Davies *et al.*, 2007), and Barrett-associated adenocarcinoma OE33 cells (Sturmeiy *et al.*, 2009). Masannat *et al.* (2009) reported no increase in the number of FPG-sensitive sites in MCF-7 cells treated with 1% methylene blue for 5 minutes in the presence of white light, but alkali-labile sites were significantly increased by this treatment, as was total DNA damage. Similar results were reported by Sturmeiy *et al.* (2009) with OE33 cells treated with methylene blue and white light (significant increase in alkali-labile sites, but not FPG-sensitive sites). In all other cell lines, DNA damage in the form of both alkali-labile sites and FPG-sensitive sites) was observed after treatment with methylene blue in the presence of white light. To determine if one particular portion of the spectrum was involved in the photoactivation of methylene blue, Sturmeiy *et al.* (2009) conducted a series of experiments using white light and filtered light to activate methylene blue and assess DNA damage levels in OE33 cells. The concentrations of methylene blue ranged from 0.015 to 15 mM (0.0005–0.5%), with the highest concentration equal to the clinically relevant concentration used in colonoscopies to visualize suspicious areas for biopsy. Only the highest concentration of methylene blue induced significant increases in DNA damage in OE33 cells with white-light activation. However, red light (580–700 nm) induced DNA damage at a lower concentration of methylene blue (1.5 mM or 0.05%) and increased the frequency of both alkali-labile sites and FPG-sensitive sites; no increases in DNA damage were seen when light was filtered to allow only the blue or the green portions of the spectrum to interact with methylene blue. Lowering the concentration of methylene blue used in the clinic, and/or eliminating the red portion of the white-light spectrum used to illuminate colonic epithelium during colonoscopy might thus result in reduction of DNA damage in sensitive tissues during these medical procedures.

(c) *Chromosomal damage*

(i) *In vitro*

The results of tests measuring induction of sister-chromatid exchange in cultured Chinese hamster lung V79 cells ([Popescu et al., 1977](#)), and Syrian hamster fibroblast (baby hamster kidney) BHK-1 cells ([MacRae et al., 1980](#)) treated with methylene blue in the absence of photoactivation were generally negative. One exception was reported, where Chinese hamster V79 cells showed significant increases in the frequency of sister-chromatid exchange in the absence, but not in the presence, of photoactivation ([Speit & Vogel, 1979](#)). No induction of chromosomal aberration was seen in Chinese hamster V79 cells treated with methylene blue in the absence of photoactivation ([Popescu et al., 1977](#)). Negative results were also reported in another test for chromosomal aberration in Chinese hamster ovary cells ([Au & Hsu, 1979](#)). [The Working Group noted that caution should be used in interpreting the results of [Au & Hsu \(1979\)](#) due to the inadequate description of the protocol and other deficiencies, including the brief exposure time and the small number of cells scored.] In a study by the National Toxicology Program ([NTP, 2008](#)), induction of sister-chromatid exchange and of chromosomal aberration with and without metabolic activation was observed in Chinese hamster ovary cells treated with methylene blue.

(ii) *In vivo*

Despite extensive evidence for mutagenicity and induction of DNA damage by methylene blue *in vitro*, particularly with white-light activation, no evidence for genotoxicity has been observed in a limited number of standard tests *in vivo*, all of which investigated some aspect of chromosomal damage. No significant increase in the frequency of sister-chromatid exchange was seen in bone-marrow cells of adult Chinese hamsters given a single intraperitoneal injection of methylene blue at 12 mg/kg bw ([Speit, 1982](#)).

Similarly, no increases in the frequency of micronucleated erythrocytes were observed in bone-marrow cells or peripheral blood erythrocytes of male B6C3F<sub>1</sub> mice given a single intraperitoneal dose of methylene blue, or in peripheral blood erythrocytes of male B6C3F<sub>1</sub> mice treated by gavage with methylene blue for 5 days per week for 3 months ([NTP, 2008](#)).

#### 4.2.3 Metabolites of methylene blue

(a) *Azure A*

Azure A was mutagenic in *Salmonella typhimurium* strains TA98 and TA100, and *Escherichia coli* strain WP2 *uvrA* pKM101, with and without exogenous metabolic activation ([NTP, 2008](#)). Azure A also induced chromosomal damage in cultured Chinese hamster ovary cells in the absence of exogenous metabolic activation at doses (10 and 20 µM) that produced marked cytotoxicity ([Au & Hsu, 1979](#)).

(b) *Azure B*

Azure B was mutagenic in *Salmonella typhimurium* strains TA98 and TA100, and *Escherichia coli* strain WP2 *uvrA* pKM101, with and without exogenous metabolic activation ([NTP, 2008](#)). Azure B also induced chromosomal damage in cultured Chinese hamster ovary cells in the absence of exogenous metabolic activation at a dose (20 µM) that produced marked cytotoxicity ([Au & Hsu, 1979](#)).

(c) *Azure C*

Azure C was mutagenic in *Salmonella typhimurium* strains TA98 and TA100, and *Escherichia coli* strain WP2 *uvrA* pKM101, with and without exogenous metabolic activation ([NTP, 2008](#)). Azure C also induced chromosomal damage in cultured Chinese hamster ovary cells in the absence of exogenous metabolic activation at a dose (20 µM) that produced marked cytotoxicity ([Au & Hsu, 1979](#)).

## 4.3 Other relevant mechanisms

### 4.3.1 General adverse effects

In humans, large intravenous doses of methylene blue (~500 mg) have been reported to cause nausea, abdominal and chest pain, cyanosis, methaemoglobinaemia, sweating, dizziness, headache, and confusion ([Clifton & Leikin, 2003](#); [Oz et al., 2011](#)). Toxicity in infants exposed to methylene blue during prenatal or perinatal diagnostic or therapeutic procedures is well documented: hyperbilirubinaemia, haemolytic anaemia, formation of Heinz bodies, erythrocytic blister cells, skin discoloration, and photosensitization are the most commonly reported adverse effects ([Sills & Zinkham, 1994](#); [Porat et al., 1996](#); [Cragan, 1999](#)).

A series of acute toxic effects have been described in animals exposed to methylene blue, including haemoconcentration, hypothermia, acidosis, hypercapnia, hypoxia, increases in blood pressure, changes in respiratory frequency and amplitude, corneal injury, conjunctival damage, and formation of Heinz bodies ([Auerbach et al., 2010](#)).

### 4.3.2 Haematological toxicity

Severe toxic methaemoglobinaemia can be treated by intravenous administration of methylene blue (1–2 mg/kg bw). In the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), the dye is converted by methaemoglobin reductases in erythrocytes to leucomethylene blue, which then reduces methaemoglobin nonenzymatically, restoring functional haemoglobin and methylene blue. This redox cycle is sustained by regeneration of NADPH via the hexose monophosphate shunt (pentose phosphate pathway). However, at higher concentrations, methylene blue oxidizes ferrous iron in haemoglobin to the ferric state, producing methaemoglobin ([Bradberry et al., 2001](#)).

Given that glucose-6-phosphate dehydrogenase is required for the enzymatic pentose phosphate pathway that produces NADPH, patients with glucose-6-phosphate dehydrogenase deficiency have depleted NADPH levels. In these patients, methylene blue may exacerbate haemolytic anaemia, and haemolysis favours the formation of methylene blue-induced methaemoglobin ([Smith & Thron, 1972](#); [Bilgin et al., 1998](#)).

A study compared the responses of several species to a single intraperitoneal injection of methylene blue (20–100 mg/kg bw in cats, dogs, and guinea-pigs; 20–200 mg/kg bw in mice, rabbits, and rats). Although the tolerance for methylene blue varied considerably, most species had a decrease in erythrocytes and haemoglobin, and an increase in reticulocytes within a few days after treatment. Cats and dogs were the most sensitive species, with Heinz bodies detected 4 and 6 hours, respectively, after administration of methylene blue. Heinz bodies were also detected in mice (100% incidence, at 200 mg/kg bw after 24 hours), rats (12% incidence, at 200 mg/kg bw after 96 hours), rabbits (70% incidence, at 200 mg/kg bw after 96 hours), and guinea-pigs (incidence was 4%, at 100 mg/kg bw, after 72 hours) ([Rentsch & Wittekind, 1967](#)).

In a 90-day study of toxicity by the NTP, methylene blue was administered at doses of 0, 25, 50, 100, and 200 mg/kg bw by gavage to F344/N rats and B6C3F<sub>1</sub> mice. The treatment resulted in methaemoglobin formation, oxidative damage to erythrocytes, and dose-related regenerative Heinz-body anaemia in rats and mice. Splenomegaly and an increase in splenic haematopoiesis occurred in treated rats and mice. Splenic congestion and bone-marrow hyperplasia were also observed in treated rats. Mice showed increased liver haematopoiesis (100 mg/kg bw and above) and an accumulation of haemosiderin in Kupffer cells (50 mg/kg bw and above). These observations suggested the development of haemolytic anaemia. There was also a dose-related increase in the reticulocyte

count in treated rats and mice, suggesting a compensatory response to anaemia ([Hejtmancik et al., 2002](#); [NTP, 2008](#)).

The haematological toxicity documented in the 90-day study by the NTP (see above) served as the basis for selecting the doses of methylene blue for a long-term bioassay (0, 5, 25, and 50 mg/kg bw per day for rats; 0, 2.5, 12.5, and 25 mg/kg bw per day for mice; 5 days per week for 2 years). Similarly to the 90-day study, development of methaemoglobinemia, formation of Heinz bodies, and macrocytic responsive anaemia were observed in treated rats, while methaemoglobinaemia and formation of Heinz bodies also occurred in treated mice ([NTP, 2008](#); [Auerbach et al., 2010](#)).

#### 4.3.3 Additional mechanisms

Amino acids can undergo photo-oxidation by methylene blue and methylene blue derivatives ([Knowles & Gurnani, 1972](#)); multiple studies have been conducted on the photoinactivation of a variety of enzymes by methylene blue (reviewed in [Moura & Cordeiro, 2003](#)).

In pharmacological studies, methylene blue (1–10  $\mu\text{M}$ ) is used routinely to inhibit soluble guanylate cyclase for the analysis of cyclic guanosine monophosphate (cGMP)-mediated processes. Methylene blue also inhibits constitutive and inducible forms of nitric oxide synthase by oxidation of ferrous iron bound to the enzyme, and inactivates nitric oxide by generation of superoxide anions (reviewed in [Oz et al., 2011](#)).

Methylene blue penetrates cellular and mitochondrial membranes, accumulates within mitochondria, and improves mitochondrial respiration at low concentrations (0.5–2  $\mu\text{M}$ ) by shuttling electrons to oxygen in the electron transport chain. When acting as an alternative electron acceptor in mitochondria, methylene blue also inhibits the production of superoxide by competing with molecular oxygen. Methylene blue has been described to increase the enzymatic

activity of cytochrome oxidase in the brain (reviewed in [Oz et al., 2009](#)).

Methylene blue and its metabolite, azure B, are reversible inhibitors of monoamine oxidase. This inhibition may underlie adverse effects, but also psycho- and neuromodulatory actions associated with methylene blue taken as a drug ([Ramsay et al., 2007](#); [Petzer et al., 2012](#)).

## 4.4 Susceptibility

No data were available to the Working Group.

## 4.5 Mechanistic considerations

Methylene blue absorbs energy directly from a light source and then transfers this energy to molecular oxygen, generating singlet oxygen ( $^1\text{O}_2$ ). Singlet oxygen is electrophilic and can oxidize electron-rich double bonds in bio(macro) molecules ([Tardivo et al., 2005](#)).

Two mechanisms of action, involving photo-activation, can also be envisaged. Excitation of methylene blue can produce both a singlet and a triplet species; the excess triplet energy can be transferred through electrons (type I mechanism) or energy (type II mechanism) ([Tardivo et al., 2005](#)). Both mechanisms can damage bio(macro) molecules. Energy transfer can cause strand breaks in nucleic acids, thereby leading to DNA damage. Electron transfer can produce reactive oxygen species, including hydroxyl radicals and hydroperoxides, which can be detrimental to the integrity of nucleic acids, proteins, and lipids.

Although the carcinogenicity of methylene blue may partly arise via photoactivation, the rodent bioassays were conducted without light activation. Therefore other mechanisms are likely to operate. It is currently unclear whether the effects of methylene blue upon enzyme-mediated processes, such as inhibition of nitric oxide synthase, with possible generation of superoxide anions, are a factor in the process.

## 5. Summary of Data Reported

### 5.1 Exposure data

Methylene blue is a synthetic chemical dye. Methylene blue has a variety of medical uses, including use as an antidote to methaemoglobinemia induced by environmental poisons such as excessive nitrate in well-water or cyanide compounds. Other indications include treatment of psychiatric disorders. Recent studies have investigated its use in Alzheimer disease and therapy for malaria. Other uses include staining in bacteriology, and uses as a redox colorimetric agent, as a contrast agent in medical procedures, as a dye, or as a disinfectant. Occupational exposure has been documented. Overall, data on exposure are limited, but substantial sales have been reported in the USA and Brazil.

### 5.2 Human carcinogenicity data

No data were available to the Working Group.

### 5.3 Animal carcinogenicity data

Methylene blue was tested for carcinogenicity in one study in mice treated by gavage for 2 years, and one study in rats treated by gavage for 2 years.

In the study in mice, methylene blue caused a significant positive trend in the incidence of carcinoma, and of adenoma or carcinoma (combined), of the small intestine in males. In males, a significant positive trend and a significant increase in the incidence of bronchiolo-alveolar carcinoma of the lung at the highest dose were considered not to be related to treatment. Treatment with methylene blue caused the incidence of malignant lymphoma in females to increase with a significant positive trend, but all incidences were well within the range for historical controls.

In the study in rats treated by gavage, methylene blue caused a significant increase in

the incidence of pancreatic islet cell adenoma in males at the intermediate dose. The incidence of pancreatic islet cell adenoma or carcinoma (combined) in males at the intermediate dose was significantly increased only as the result of the increased incidence of adenoma; the incidence of carcinoma was within the range for historical controls. No significant increase in the incidence of any neoplasm was observed in females.

### 5.4 Mechanistic and other relevant data

Methylene blue is well absorbed, reduced, and is excreted largely in the urine as the reduced form, leucomethylene blue.

Methylene blue and its *N*-demethylated metabolites, azure A, azure B, and azure C, have given positive results in an extensive series of standard in-vitro assays for genotoxicity, both in the absence and presence of exogenous metabolic activation.

At high doses, methylene blue oxidizes ferrous iron in haemoglobin to the ferric state, producing methaemoglobin. Exposure to methylene blue results in haematological toxicity, including formation of Heinz bodies and haemolytic anaemia, in several species.

Photoactivation of methylene blue produces high-energy species that have the potential to damage DNA, proteins, and lipids, either directly or through the production of reactive oxygen species. In the absence of light activation, the carcinogenicity of methylene blue is likely to arise from other mechanisms. A potential mechanism is the inhibition of nitric oxide synthase, with possible generation of superoxide anions.



## 6. Evaluation

### 6.1 Cancer in humans

No data were available to the Working Group.

### 6.2 Cancer in experimental animals

There is *limited evidence* for the carcinogenicity of methylene blue in experimental animals.

### 6.3 Overall evaluation

Methylene blue is *not classifiable as to its carcinogenicity in humans (Group 3)*.

## References

- Aeschlimann C, Cerny T, Küpfer A (1996). Inhibition of (mono)amine oxidase activity and prevention of ifosfamide encephalopathy by methylene blue. *Drug Metab Dispos*, 24:1336–1339. PMID:[8971139](#)
- ATSDR (1992). Toxicological profile for nitrophenols. Atlanta (GA): Agency for Toxic Substances and Disease Registry, United States Public Health Service.
- Au W, Hsu TC (1979). Studies on the clastogenic effects of biologic stains and dyes. *Environ Mutagen*, 1:27–35. doi:[10.1002/em.2860010109](#) PMID:[95447](#)
- Auerbach SS, Bristol DW, Peckham JC *et al.* (2010). Toxicity and carcinogenicity studies of methylene blue trihydrate in F344N rats and B6C3F1 mice. *Food Chem Toxicol*, 48:169–177. doi:[10.1016/j.fct.2009.09.034](#) PMID:[19804809](#)
- Belaz-David N, Decosterd LA, Appenzeller M *et al.* (1997). Spectrophotometric determination of methylene blue in biological fluids after ion-pair extraction and evidence of its adsorption on plastic polymers. *Eur J Pharm Sci*, 5:335–345. doi:[10.1016/S0928-0987\(97\)00061-4](#)
- Berneth H (2008). Azine dyes. In: Ullmann's Encyclopedia of Industrial Chemistry. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, pp. 475–514. doi:[10.1002/14356007.a03\\_213.pub3](#).
- Bilgin H, Özcan B, Bilgin T (1998). Methemoglobinemia induced by methylene blue perturbation during laparoscopy. *Acta Anaesthesiol Scand*, 42:594–595. doi:[10.1111/j.1399-6576.1998.tb05173.x](#) PMID:[9605379](#)
- Borwitzky H, Haefeli WE, Burhenne J (2005). Analysis of methylene blue in human urine by capillary electrophoresis. *J Chromatogr B Analyt Technol Biomed Life Sci*, 826:244–251. doi:[10.1016/j.jchromb.2005.09.013](#) PMID:[16182616](#)
- Bountogo M, Zoungrana A, Coulibaly B *et al.* (2010). Efficacy of methylene blue monotherapy in semi-immune adults with uncomplicated falciparum malaria: a controlled trial in Burkina Faso. *Trop Med Int Health*, 15:713–717. doi:[10.1111/j.1365-3156.2010.02526.x](#) PMID:[20374561](#)
- Bradberry SM, Aw T-C, Williams NR, Vale JA (2001). Occupational methaemoglobinemia. *Occup Environ Med*, 58:611–615, quiz 616. doi:[10.1136/oem.58.9.611](#) PMID:[11511749](#)
- Brendel M (1973). Different photodynamic action of proflavine and methylene blue on bacteriophage. II. Mutation induction in extracellularly treated *Serratia* phage kappa. *Mol Gen Genet*, 120:171–180. doi:[10.1007/BF00267245](#) PMID:[4568530](#)
- British Pharmacopoeia Commission (2005). British Pharmacopoeia 2005. London: Medicines and Healthcare products Regulatory Agency.
- Burhenne J, Riedel KD, Rengelshausen J *et al.* (2008). Quantification of cationic anti-malaria agent methylene blue in different human biological matrices using cation exchange chromatography coupled to tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*, 863:273–282. doi:[10.1016/j.jchromb.2008.01.028](#) PMID:[18258499](#)
- ChemNet (2013). Methylene blue. Royal Society of Chemistry. Available from: <http://chemnet.rsc.org>.
- Christensen CM, Farrar HC, Kearns GL (1996). Protracted methemoglobinemia after phenazopyridine overdose in an infant. *J Clin Pharmacol*, 36:112–116. doi:[10.1002/j.1552-4604.1996.tb04175.x](#) PMID:[8852386](#)
- Chung KT, Fulk GE, Andrews AW (1981). Mutagenicity testing of some commonly used dyes. *Appl Environ Microbiol*, 42:641–648. PMID:[7039509](#)
- Clark AM (1953). Mutagenic activity of dyes in *Drosophila melanogaster*. *Am Nat*, 87:295–305. doi:[10.1086/281787](#)
- Clifton J 2nd, Leikin JB (2003). Methylene blue. *Am J Ther*, 10:289–291. doi:[10.1097/00045391-200307000-00009](#) PMID:[12845393](#)
- Coulibaly B, Zoungrana A, Mockenhaupt FP *et al.* (2009). Strong gametocytocidal effect of methylene blue-based combination therapy against falciparum malaria: a randomised controlled trial. *PLoS ONE*, 4:e5318 doi:[10.1371/journal.pone.0005318](#) PMID:[19415120](#)
- Cragan JD (1999). Teratogen update: methylene blue. *Teratology*, 60:42–48. doi:[10.1002/\(SICI\)1096-9926\(199907\)60:1<42::AID-TERA12>3.0.CO;2-Z](#) PMID:[10413340](#)
- Davies J, Burke D, Olliver JR *et al.* (2007). Methylene blue but not indigo carmine causes DNA damage to colonocytes *in vitro* and *in vivo* at concentrations

- used in clinical chromoendoscopy. *Gut*, 56:155–156. doi:[10.1136/gut.2006.107300](https://doi.org/10.1136/gut.2006.107300) PMID:[17172595](https://pubmed.ncbi.nlm.nih.gov/17172595/)
- DiSanto AR, Wagner JG (1972). Pharmacokinetics of highly ionized drugs. I. Methylene blue–whole blood, urine, and tissue assays. *J Pharm Sci*, 61(4):598–602. doi:[10.1002/jps.2600610422](https://doi.org/10.1002/jps.2600610422) PMID:[5014319](https://pubmed.ncbi.nlm.nih.gov/5014319/)
- DiSanto AR, Wagner JG (1972a). Pharmacokinetics of highly ionized drugs. II. Methylene blue–absorption, metabolism, and excretion in man and dog after oral administration. *J Pharm Sci*, 61:1086–1090. doi:[10.1002/jps.2600610710](https://doi.org/10.1002/jps.2600610710) PMID:[5044807](https://pubmed.ncbi.nlm.nih.gov/5044807/)
- DiSanto AR, Wagner JG (1972b). Pharmacokinetics of highly ionized drugs. I. Methylene blue–whole blood, urine, and tissue assays. *J Pharm Sci*, 61:598–602. doi:[10.1002/jps.2600610422](https://doi.org/10.1002/jps.2600610422) PMID:[5014319](https://pubmed.ncbi.nlm.nih.gov/5014319/)
- DiSanto AR, Wagner JG (1972c). Pharmacokinetics of highly ionized drugs. III. Methylene blue–blood levels in the dog and tissue levels in the rat following intravenous administration. *J Pharm Sci*, 61:1090–1094. doi:[10.1002/jps.2600610711](https://doi.org/10.1002/jps.2600610711) PMID:[5044808](https://pubmed.ncbi.nlm.nih.gov/5044808/)
- EDQM (2008). Methylthionium chloride. In: European Pharmacopoeia. Strasbourg, France: European Directorate for the Quality of Medicines & HealthCare.
- Ehrlich P (1881). Ueber das Methylenblau und seine klinisch-bakterioskopische Verwerthung. *Z Klin Med*, 2:710–713. [German]
- Epe B, Hegler J, Wild D (1989). Singlet oxygen as an ultimately reactive species in *Salmonella typhimurium* DNA damage induced by methylene blue/visible light. *Carcinogenesis*, 10:2019–2024. doi:[10.1093/carcin/10.11.2019](https://doi.org/10.1093/carcin/10.11.2019) PMID:[2680144](https://pubmed.ncbi.nlm.nih.gov/2680144/)
- Epe B, Mützel P, Adam W (1988). DNA damage by oxygen radicals and excited state species: a comparative study using enzymatic probes *in vitro*. *Chem Biol Interact*, 67:149–165. doi:[10.1016/0009-2797\(88\)90094-4](https://doi.org/10.1016/0009-2797(88)90094-4) PMID:[2844422](https://pubmed.ncbi.nlm.nih.gov/2844422/)
- Epe B, Pflaum M, Boiteux S (1993). DNA damage induced by photosensitizers in cellular and cell-free systems. *Mutat Res*, 299:135–145. doi:[10.1016/0165-1218\(93\)90091-Q](https://doi.org/10.1016/0165-1218(93)90091-Q) PMID:[7683082](https://pubmed.ncbi.nlm.nih.gov/7683082/)
- Eroglu L, Cağlayan B (1997). Anxiolytic and antidepressant properties of methylene blue in animal models. *Pharmacol Res*, 36:381–385. doi:[10.1006/phrs.1997.0245](https://doi.org/10.1006/phrs.1997.0245) PMID:[9441729](https://pubmed.ncbi.nlm.nih.gov/9441729/)
- FDA (2011). Drug Safety Communication: Serious CNS reactions possible when methylene blue is given to patients taking certain psychiatric medications. Safety announcement dated 26 July 2011. Silver Spring (MD): United States Food and Drug Administration. Available from: <http://www.fda.gov/Drugs/DrugSafety/ucm263190.htm>, accessed 1 October 2014.
- Gaudette NF, Lodge JW (2005). Determination of methylene blue and leucomethylene blue in male and female Fischer 344 rat urine and B6C3F<sub>1</sub> mouse urine. *J Anal Toxicol*, 29:28–33. doi:[10.1093/jat/29.1.28](https://doi.org/10.1093/jat/29.1.28) PMID:[15808010](https://pubmed.ncbi.nlm.nih.gov/15808010/)
- Gutter B, Speck WT, Rosenkranz HS (1977). A study of the photoinduced mutagenicity of methylene blue. *Mutat Res*, 44:177–181. doi:[10.1016/0027-5107\(77\)90075-6](https://doi.org/10.1016/0027-5107(77)90075-6) PMID:[331101](https://pubmed.ncbi.nlm.nih.gov/331101/)
- Guttman P, Ehrlich P (1891). Ueber die Wirkung des Methylenblau bei Malaria. *Berl Klin Wochenschr*, 28:953–956. [German]
- Hejtmančík MR, Ryan MJ, Toft JD *et al.* (2002). Hematological effects in F344 rats and B6C3F<sub>1</sub> mice during the 13-week gavage toxicity study of methylene blue trihydrate. *Toxicol Sci*, 65:126–134. doi:[10.1093/toxsci/65.1.126](https://doi.org/10.1093/toxsci/65.1.126) PMID:[11752692](https://pubmed.ncbi.nlm.nih.gov/11752692/)
- Horváthová E, Kozics K, Srančíková A *et al.* (2012). Borneol administration protects primary rat hepatocytes against exogenous oxidative DNA damage. *Mutagenesis*, 27:581–588. doi:[10.1093/mutage/ges023](https://doi.org/10.1093/mutage/ges023) PMID:[22544524](https://pubmed.ncbi.nlm.nih.gov/22544524/)
- IMS Health (2012). Multinational Integrated Data Analysis (MIDAS). IMS Health, Plymouth Meeting, 2012, Pennsylvania, USA.
- Ito T, Kobayashi K (1977). A survey of *in vivo* photo-dynamic activity of xanthenes, thiazines, and acridines in yeast cells. *Photochem Photobiol*, 26:581–587. doi:[10.1111/j.1751-1097.1977.tb07536.x](https://doi.org/10.1111/j.1751-1097.1977.tb07536.x)
- Kasuga Y, Hishida M, Tanahashi N (1991). Simultaneous determination of malachite green and methylene blue in cultured fishes by high performance liquid chromatography. *Shokuhin Eiseigaku Zasshi*, 32:137–141. doi:[10.3358/shokueishi.32.137](https://doi.org/10.3358/shokueishi.32.137)
- Kimoto K, Gohda R, Murayama K *et al.* (1996). Sensitive detection of near-infrared fluorescent dyes using high-performance liquid chromatography with peroxoxalate chemiluminescence detection system. *Biomed Chromatogr*, 10:189–190. doi:[10.1002/\(SICI\)1099-0801\(199607\)10:4<189::AID-BMC585>3.0.CO;2-P](https://doi.org/10.1002/(SICI)1099-0801(199607)10:4<189::AID-BMC585>3.0.CO;2-P) PMID:[8831965](https://pubmed.ncbi.nlm.nih.gov/8831965/)
- Knowles A, Gurnani S (1972). A study of the methylene blue-sensitized oxidation of amino acids. *Photochem Photobiol*, 16:95–108. doi:[10.1111/j.1751-1097.1972.tb07341.x](https://doi.org/10.1111/j.1751-1097.1972.tb07341.x) PMID:[5052681](https://pubmed.ncbi.nlm.nih.gov/5052681/)
- Kosswig K (2000). Surfactants. In: Ullmann's Encyclopedia of Industrial Chemistry. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, pp. 487–505. doi:[10.1002/14356007.a25\\_747](https://doi.org/10.1002/14356007.a25_747)
- Kozaki A, Watanabe J (1981). Dose dependency of apparent volumes of distribution for methylene blue in rabbits. *J Pharmacobiodyn*, 4:49–57. doi:[10.1248/bpb1978.4.49](https://doi.org/10.1248/bpb1978.4.49) PMID:[7277192](https://pubmed.ncbi.nlm.nih.gov/7277192/)
- Laassis B, Aaron J-J, Mahedero MC (1994). Fluorimetric determination of phenothiazine derivatives by photooxidation in a flow-injection system. *Talanta*, 41:1985–1989. doi:[10.1016/0039-9140\(94\)00162-6](https://doi.org/10.1016/0039-9140(94)00162-6) PMID:[18966160](https://pubmed.ncbi.nlm.nih.gov/18966160/)
- Lábaj J, Slamenová D, Lazarová M, Kosíková B (2007). Induction of DNA-lesions in freshly isolated rat hepatocytes by different genotoxins and their

- reduction by lignin given either as a dietary component or in *in vitro* conditions. *Nutr Cancer*, 57:209–215. doi:[10.1080/01635580701277643](https://doi.org/10.1080/01635580701277643) PMID:[17571955](https://pubmed.ncbi.nlm.nih.gov/17571955/)
- Lee CH, Chang CT, Wetmur JG (1973). Induced circular dichroism of DNA-dye complexes. *Biopolymers*, 12:1099–1122. doi:[10.1002/bip.1973.360120514](https://doi.org/10.1002/bip.1973.360120514) PMID:[4710250](https://pubmed.ncbi.nlm.nih.gov/4710250/)
- Lunn G, Sansone EB (1991). Decontamination of aqueous solutions of biological stains. *Biotech Histochem*, 66:307–315. doi:[10.3109/10520299109109992](https://doi.org/10.3109/10520299109109992) PMID:[1725856](https://pubmed.ncbi.nlm.nih.gov/1725856/)
- MacRae WD, Chan GF, Wat CK *et al.* (1980). Examination of naturally occurring polyacetylenes and alpha-terthienyl for their ability to induce cytogenetic damage. *Experientia*, 36:1096–1097. doi:[10.1007/BF01965990](https://doi.org/10.1007/BF01965990) PMID:[7418849](https://pubmed.ncbi.nlm.nih.gov/7418849/)
- Masannat YA, Hanby A, Horgan K, Hardie LJ (2009). DNA damaging effects of the dyes used in sentinel node biopsy: possible implications for clinical practice. *J Surg Res*, 154:234–238. doi:[10.1016/j.jss.2008.07.039](https://doi.org/10.1016/j.jss.2008.07.039) PMID:[19181339](https://pubmed.ncbi.nlm.nih.gov/19181339/)
- McBride TJ, Schneider JE, Floyd RA, Loeb LA (1992). Mutations induced by methylene blue plus light in single-stranded M13mp2. *Proc Natl Acad Sci USA*, 89:6866–6870. doi:[10.1073/pnas.89.15.6866](https://doi.org/10.1073/pnas.89.15.6866) PMID:[1495976](https://pubmed.ncbi.nlm.nih.gov/1495976/)
- McCarroll NE, Piper CE, Keech BH (1981). An *E. coli* micro-suspension assay for the detection of DNA damage induced by direct-acting agents and promutagens. *Environ Mutagen*, 3:429–444. doi:[10.1002/em.2860030404](https://doi.org/10.1002/em.2860030404) PMID:[7021147](https://pubmed.ncbi.nlm.nih.gov/7021147/)
- Medscape (2013). Methylene blue (Rx). Dosing and uses. Available from: <http://reference.medscape.com/drug/methylene-blue-343739>, accessed 5 June 2013.
- Mohn GR, Kerklaan PR, van Zeeland AA *et al.* (1984). Methodologies for the determination of various genetic effects in permeable strains of *E. coli* K-12 differing in DNA repair capacity. Quantification of DNA adduct formation, experiments with organ homogenates and hepatocytes, and animal-mediated assays. *Mutat Res*, 125:153–184. doi:[10.1016/0027-5107\(84\)90067-8](https://doi.org/10.1016/0027-5107(84)90067-8) PMID:[6230533](https://pubmed.ncbi.nlm.nih.gov/6230533/)
- Moura JC, Cordeiro N (2003). 3,7-Bis(dialkylamino)phenothiazin-5-ium derivatives: biomedical applications and biological activity. *Curr Drug Targets*, 4:133–141. doi:[10.2174/1389450033346902](https://doi.org/10.2174/1389450033346902) PMID:[12558066](https://pubmed.ncbi.nlm.nih.gov/12558066/)
- Munns RK, Holland DC, Roybal JE *et al.* (1992). Liquid chromatographic determination of methylene blue and its metabolites in milk. *J AOAC Int*, 75:796–800.
- Naylor GJ, Martin B, Hopwood SE, Watson Y (1986). A two-year double-blind crossover trial of the prophylactic effect of methylene blue in manic-depressive psychosis. *Biol Psychiatry*, 21:915–920. doi:[10.1016/0006-3223\(86\)90265-9](https://doi.org/10.1016/0006-3223(86)90265-9) PMID:[3091097](https://pubmed.ncbi.nlm.nih.gov/3091097/)
- Nordén B, Tjerneld F (1982). Structure of methylene blue-DNA complexes studied by linear and circular dichroism spectroscopy. *Biopolymers*, 21:1713–1734. doi:[10.1002/bip.360210904](https://doi.org/10.1002/bip.360210904) PMID:[7126754](https://pubmed.ncbi.nlm.nih.gov/7126754/)
- NTP (2008). Toxicology and carcinogenesis studies of methylene blue trihydrate (Cas No. 7220–79–3) in F344/N rats and B6C3F1 mice (gavage studies). *Natl Toxicol Program Tech Rep Ser*, 540:1–224. PMID:[18685714](https://pubmed.ncbi.nlm.nih.gov/18685714/)
- O’Neil MJ, Heckelman PE, Koch CB *et al.* (2006). The Merck Index: an encyclopedia of chemicals, drugs, and biologicals, 14th Edition (Version 14.6). Whitehouse Station (NJ): Merck & Co., Inc.
- Olliver JR, Wild CP, Sahay P *et al.* (2003). Chromoendoscopy with methylene blue and associated DNA damage in Barrett’s oesophagus. *Lancet*, 362:373–374. doi:[10.1016/S0140-6736\(03\)14026-3](https://doi.org/10.1016/S0140-6736(03)14026-3) PMID:[12907012](https://pubmed.ncbi.nlm.nih.gov/12907012/)
- Onur F, Acar N (1992). Simultaneous determination of methylene blue, hexamethylene tetramine and resorcinol in pharmaceutical formulations by first-derivative UV spectrophotometry. *Int J Pharm*, 78:89–91. doi:[10.1016/0378-5173\(92\)90359-A](https://doi.org/10.1016/0378-5173(92)90359-A)
- Oz M, Lorke DE, Hasan M, Petroianu GA (2011). Cellular and molecular actions of Methylene Blue in the nervous system. *Med Res Rev*, 31:93–117. doi:[10.1002/med.20177](https://doi.org/10.1002/med.20177) PMID:[19760660](https://pubmed.ncbi.nlm.nih.gov/19760660/)
- Oz M, Lorke DE, Petroianu GA (2009). Methylene blue and Alzheimer’s disease. *Biochem Pharmacol*, 78:927–932. doi:[10.1016/j.bcp.2009.04.034](https://doi.org/10.1016/j.bcp.2009.04.034) PMID:[19433072](https://pubmed.ncbi.nlm.nih.gov/19433072/)
- Peter C, Hongwan D, Küpfer A, Lauterburg BH (2000). Pharmacokinetics and organ distribution of intravenous and oral methylene blue. *Eur J Clin Pharmacol*, 56:247–250. doi:[10.1007/s002280000124](https://doi.org/10.1007/s002280000124) PMID:[10952480](https://pubmed.ncbi.nlm.nih.gov/10952480/)
- Petzer A, Harvey BH, Wegener G, Petzer JP (2012). Azure B, a metabolite of methylene blue, is a high-potency, reversible inhibitor of monoamine oxidase. *Toxicol Appl Pharmacol*, 258:403–409. doi:[10.1016/j.taap.2011.12.005](https://doi.org/10.1016/j.taap.2011.12.005) PMID:[22197611](https://pubmed.ncbi.nlm.nih.gov/22197611/)
- Popescu NC, Turnbull D, DiPaolo JA (1977). Sister chromatid exchange and chromosome aberration analysis with the use of several carcinogens and noncarcinogens. *J Natl Cancer Inst*, 59:289–293. PMID:[406414](https://pubmed.ncbi.nlm.nih.gov/406414/)
- Porat R, Gilbert S, Magilner D (1996). Methylene blue-induced phototoxicity: an unrecognized complication. *Pediatrics*, 97:717–721. PMID:[8628613](https://pubmed.ncbi.nlm.nih.gov/8628613/)
- Pruthi S, Haakenson C, Brost BC *et al.* (2011). Pharmacokinetics of methylene blue dye for lymphatic mapping in breast cancer-implications for use in pregnancy. *Am J Surg*, 201:70–75. doi:[10.1016/j.amjsurg.2009.03.013](https://doi.org/10.1016/j.amjsurg.2009.03.013) PMID:[21167367](https://pubmed.ncbi.nlm.nih.gov/21167367/)
- PubChem (2013). Methylene blue. Pubchem database. National Center for Biotechnology Information. Available from: <https://pubchem.ncbi.nlm.nih.gov/> [online database].
- Rager T, Geoffroy A’, Hilfiker R, Storey JMD (2012). The crystalline state of methylene blue: a zoo of hydrates.

- Phys Chem Chem Phys*, 14:8074–8082. doi:[10.1039/c2cp40128b](https://doi.org/10.1039/c2cp40128b) PMID:[22481217](https://pubmed.ncbi.nlm.nih.gov/22481217/)
- Ramsay RR, Dunford C, Gillman PK (2007). Methylene blue and serotonin toxicity: inhibition of monoamine oxidase A (MAO A) confirms a theoretical prediction. *Br J Pharmacol*, 152:946–951. doi:[10.1038/sj.bjp.0707430](https://doi.org/10.1038/sj.bjp.0707430) PMID:[17721552](https://pubmed.ncbi.nlm.nih.gov/17721552/)
- Rengelshausen J, Burhenne J, Fröhlich M *et al.* (2004). Pharmacokinetic interaction of chloroquine and methylene blue combination against malaria. *Eur J Clin Pharmacol*, 60:709–715. doi:[10.1007/s00228-004-0818-0](https://doi.org/10.1007/s00228-004-0818-0) PMID:[15619134](https://pubmed.ncbi.nlm.nih.gov/15619134/)
- Rentsch G, Wittekind D (1967). Methylene blue and erythrocytes in the living animal. Contribution to the toxicology of methylene blue and formation of Heinz bodies. *Toxicol Appl Pharmacol*, 11:81–87. doi:[10.1016/0041-008X\(67\)90029-4](https://doi.org/10.1016/0041-008X(67)90029-4) PMID:[6056158](https://pubmed.ncbi.nlm.nih.gov/6056158/)
- Roybal JE, Munns RK, Hurlbut JA, Shimoda W (1989). High-performance liquid chromatography of gentian violet, its demethylated metabolites, leucogentian violet and methylene blue with electrochemical detection. *J Chromatogr*, 467:259–266. doi:[10.1016/S0021-9673\(01\)93970-6](https://doi.org/10.1016/S0021-9673(01)93970-6) PMID:[2753937](https://pubmed.ncbi.nlm.nih.gov/2753937/)
- Roybal JE, Pfenning AP, Turnipseed SB *et al.* (1996). Dye residues in foods of animal origin. ACS Symposium Series, 636: 169–184. doi:[10.1021/bk-1996-0636-ch018](https://doi.org/10.1021/bk-1996-0636-ch018)
- Sabnis RW, Ross E, Köthe J *et al.* (2009). Indicator reagents. In: Ullmann's Encyclopedia of Industrial Chemistry. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, pp. 9–53. doi:[10.1002/14356007.a14\\_127.pub2](https://doi.org/10.1002/14356007.a14_127.pub2)
- Schirmer RH, Adler H, Pickhardt M, Mandelkow E (2011). “Lest we forget you - methylene blue...” *Neurobiol Aging*, 32:2325.e7–2325.e16. doi:[10.1016/j.neurobiolaging.2010.12.012](https://doi.org/10.1016/j.neurobiolaging.2010.12.012) PMID:[21316815](https://pubmed.ncbi.nlm.nih.gov/21316815/)
- Sills MR, Zinkham WH (1994). Methylene blue-induced Heinz body hemolytic anemia *Arch Pediatr Adolesc Med*, 148:306–310. doi:[10.1001/archpedi.1994.02170030076017](https://doi.org/10.1001/archpedi.1994.02170030076017) PMID:[8130867](https://pubmed.ncbi.nlm.nih.gov/8130867/)
- Smijs TG, Nivard MJ, Schuitmaker HJ (2004). Development of a test system for mutagenicity of photosensitizers using *Drosophila melanogaster*. *Photochem Photobiol*, 79:332–338. doi:[10.1562/2003-12-03-RA.1](https://doi.org/10.1562/2003-12-03-RA.1) PMID:[15137509](https://pubmed.ncbi.nlm.nih.gov/15137509/)
- Smith RP, Thron CD (1972). Hemoglobin, methylene blue and oxygen interactions in human red cells. *J Pharmacol Exp Ther*, 183:549–558. PMID:[4636392](https://pubmed.ncbi.nlm.nih.gov/4636392/)
- Speit G (1982). Intercalating substances do not induce sister-chromatid exchanges (SCEs) *in vivo*. *Mutat Res*, 104:261–266. doi:[10.1016/0165-7992\(82\)90154-3](https://doi.org/10.1016/0165-7992(82)90154-3) PMID:[7110164](https://pubmed.ncbi.nlm.nih.gov/7110164/)
- Speit G, Vogel W (1979). The effect on sister-chromatid exchanges of drugs and dyes by intercalation and photoactivation. *Mutat Res*, 59:223–229. doi:[10.1016/0027-5107\(79\)90161-1](https://doi.org/10.1016/0027-5107(79)90161-1) PMID:[35743](https://pubmed.ncbi.nlm.nih.gov/35743/)
- Sturmey RG, Wild CP, Hardie LJ (2009). Removal of red light minimizes methylene blue-stimulated DNA damage in oesophageal cells: implications for chromoendoscopy. *Mutagenesis*, 24:253–258. doi:[10.1093/mutage/geb004](https://doi.org/10.1093/mutage/geb004) PMID:[19218330](https://pubmed.ncbi.nlm.nih.gov/19218330/)
- Tarbin JA, Chan D, Stubbings G, Sharman M (2008). Multiresidue determination of triarylmethane and phenothiazine dyes in fish tissues by LC-MS/MS. *Anal Chim Acta*, 625:188–194. doi:[10.1016/j.aca.2008.07.018](https://doi.org/10.1016/j.aca.2008.07.018) PMID:[18724993](https://pubmed.ncbi.nlm.nih.gov/18724993/)
- Tardivo JP, Del Giglio A, de Oliveira CS, Gabrielli DS, Junqueira HC, Tada DB *et al.* (2005). Methylene blue in photodynamic therapy: From basic mechanisms to clinical applications. *Photodiagn Photodyn Ther*, 2(3):175–91. doi:[10.1016/S1572-1000\(05\)00097-9](https://doi.org/10.1016/S1572-1000(05)00097-9) PMID:[25048768](https://pubmed.ncbi.nlm.nih.gov/25048768/)
- Tuite MF, Mundy CR, Cox BS (1981). Agents that cause a high frequency of genetic change from [*psi*<sup>+</sup>] to [*psi*<sup>-</sup>] in *Saccharomyces cerevisiae*. *Genetics*, 98:691–711. PMID:[7037537](https://pubmed.ncbi.nlm.nih.gov/7037537/)
- US Pharmacopeial Convention (2013). *Methylene blue*. United States Pharmacopeia, USP36. Rockville (MD): The United States Pharmacopeial Convention.
- Villanueva A, Cañete M, Trigueros C *et al.* (1993). Photodynamic induction of DNA-protein cross-linking in solution by several sensitizers and visible light. *Biopolymers*, 33:239–244. doi:[10.1002/bip.360330206](https://doi.org/10.1002/bip.360330206) PMID:[8485298](https://pubmed.ncbi.nlm.nih.gov/8485298/)
- Walter-Sack I, Rengelshausen J, Oberwittler H, Burhenne J, Mueller O, Meissner P *et al.* (2009). High absolute bioavailability of methylene blue given as an aqueous oral formulation. *Eur J Clin Pharmacol*, 65(2):179–89. doi:[10.1007/s00228-008-0563-x](https://doi.org/10.1007/s00228-008-0563-x) PMID:[18810398](https://pubmed.ncbi.nlm.nih.gov/18810398/)
- Warth A, Goepfert B, Bopp C *et al.* (2009). Turquoise to dark green organs at autopsy. *Virchows Arch*, 454:341–344. doi:[10.1007/s00428-009-0734-x](https://doi.org/10.1007/s00428-009-0734-x) PMID:[19189125](https://pubmed.ncbi.nlm.nih.gov/19189125/)
- Webb RB, Hass BS (1984). Biological effects of dyes on bacteria. VI. Mutation induction by acridine orange and methylene blue in the dark with special reference to *Escherichia coli* WP6 (*polA1*). *Mutat Res*, 137:1–6. doi:[10.1016/0165-1218\(84\)90105-8](https://doi.org/10.1016/0165-1218(84)90105-8) PMID:[6379434](https://pubmed.ncbi.nlm.nih.gov/6379434/)
- WHO (2011). The International Pharmacopoeia, Fourth Edition. Geneva, Switzerland: World Health Organization. Available from: <http://apps.who.int/phint/en/p/about/>.
- Xu JZ, Dai L, Wu B *et al.* (2009). Determination of methylene blue residues in aquatic products by liquid chromatography-tandem mass spectrometry. *J Sep Sci*, 32::4193–4199. doi:[10.1002/jssc.200900364](https://doi.org/10.1002/jssc.200900364) PMID:[20066681](https://pubmed.ncbi.nlm.nih.gov/20066681/)
- Yamaguchi T (1981). Mutagenicity of low Molecular substances in various superoxide generating systems. *Agric Biol Chem*, 45:327–330. doi:[10.1271/bbb1961.45.327](https://doi.org/10.1271/bbb1961.45.327)
- Yang F, Xia S, Liu Z *et al.* (2011). Analysis of methylene blue and its metabolites in blood by capillary electrophoresis/electrospray ionization mass

spectrometry. *Electrophoresis*, 32:659–664. doi:[10.1002/elps.201000514](https://doi.org/10.1002/elps.201000514) PMID:[21328395](https://pubmed.ncbi.nlm.nih.gov/21328395/)

Ziv G, Heavner JE (1984). Permeability of the blood-milk barrier to methylene blue in cows and goats. *J Vet Pharmacol Ther*, 7:55–59. doi:[10.1111/j.1365-2885.1984.tb00879.x](https://doi.org/10.1111/j.1365-2885.1984.tb00879.x) PMID:[6708167](https://pubmed.ncbi.nlm.nih.gov/6708167/)

Zoungrana A, Coulibaly B, Sié A *et al.* (2008). Safety and efficacy of methylene blue combined with artesunate or amodiaquine for uncomplicated falciparum malaria: a randomized controlled trial from Burkina Faso. *PLoS ONE*, 3:e1630 doi:[10.1371/journal.pone.0001630](https://doi.org/10.1371/journal.pone.0001630) PMID:[18286187](https://pubmed.ncbi.nlm.nih.gov/18286187/)

