OFFICE OF ENVIRONMENTAL HEALTH HAZARD ASSESSMENT

Proposition 65

Evidence on the Carcinogenicity of Gentian Violet

UPDATE
January 2019

Reproductive and Cancer Hazard Assessment Branch
Office of Environmental Health Hazard Assessment
California Environmental Protection Agency

The Office of Environmental Health Hazard Assessment's (OEHHA) Reproductive and Cancer Hazard Assessment Branch was responsible for the preparation of this document.

Authors
Meng Sun, Ph.D., M.S.
Staff Toxicologist

Karin Ricker, Ph.D.
Staff Toxicologist

Gwendolyn Osborne, M.D., M.P.H.
Staff Toxicologist

M. Elizabeth Marder, Ph.D.
Environmental Scientist

Rose Schmitz, M.S.
Research Scientist III

Internal OEHHA Reviewers
Martha S. Sandy, Ph.D., M.P.H.
Chief, Reproductive and Cancer Hazard Assessment Branch

Allan Hirsch
Chief Deputy Director

Director
Lauren Zeise, Ph.D.
Office of Environmental Health Hazard Assessment

PREFACE

Proposition 65[1] requires the publication of a list of chemicals "known to the state" to cause cancer or reproductive toxicity. The Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency maintains this list in its role as lead agency for implementing Proposition 65. The Carcinogen Identification Committee (CIC) advises and assists OEHHA in compiling the list of chemicals that cause cancer as required by Health and Safety Code section 25249.8. The Committee serves as the state's qualified experts for determining whether a chemical has been clearly shown to cause cancer.

In 2010, OEHHA brought gentian violet to the CIC for prioritization and ranking for future listing consideration. In 2018, OEHHA selected gentian violet for consideration for listing by the CIC. Upon selection, the public was given the opportunity to submit information relevant to the assessment of the evidence on its carcinogenicity. No submissions were received.

This document was released for public comment on August 17, 2018. No comments were received from the public. The present document is essentially the same as the one released, with the following minor clarifications. The document as first released indicated that gentian violet is also known as "crystal violet." A statement to this effect has now been added to the Executive Summary. Figure 2 (showing the metabolism of gentian violet) has been updated to show the chemical structure of a nitrogen-centered free radical. Finally, clarifications have been added that lesions diagnosed in the past as type A reticulum cell sarcomas are likely currently classified as histiocytic sarcomas.

At its November 1, 2018 meeting, the CIC by a unanimous vote found that gentian violet had been "clearly shown through scientifically valid testing according to generally accepted principles to cause cancer." Accordingly, gentian violet (crystal violet) has been added to the Proposition 65 list of chemicals known to the state to cause cancer, effective November 23, 2018.

[1] The Safe Drinking Water and Toxic Enforcement Act of 1986 (California Health and Safety Code 25249.5 *et seq*.)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Acronyms and Abbreviations

ADI	Acceptable daily intake
AKA	Also known as
AR	Androgen receptor
CA	Chromosomal aberration
CFR	Code of Federal Regulations
CHO	Chinese hamster ovary
CYP450	Cytochrome P450
ERα	Estrogen receptor alpha
FDA	Food and Drug Administration
F344	Fischer 344
GAO	Government Accountability Office
GPCR	G protein-coupled receptor
GSH	Glutathione
HNF4A	Hepatocyte nuclear factor 4; alpha
HRP	Horseradish peroxidase
HSF1	Heat shock transcription factor 1
HTS	High-throughput screening
IARC	International Agency for Research on Cancer
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MN	Micronuclei
MNCL	Mononuclear cell leukemia
MTF1	Metal-regulatory transcription factor 1
NCI	National Cancer Institute
NCTR	National Center for Toxicological Research
NFE2L2 or Nrf2	Nuclear factor erythroid 2-like 2
NK	Natural killer
NR1H4	Nuclear receptor subfamily 1, group H, member 4
NR1I2	Nuclear receptor subfamily 1, group I, member 2
NR3C1	Nuclear receptor subfamily 3, group C, member 1
NTP	National Toxicology Program
PPARγ	Peroxisome proliferator-activated receptor gamma
ROS	Reactive oxygen species
SCE	Sister chromatid exchange
THRB	Thyroid hormone receptor, beta
UDS	Unscheduled DNA synthesis
US EPA	United States Environmental Protection Agency
VDR	Vitamin D receptor

1. EXECUTIVE SUMMARY

Gentian violet (also known as crystal violet) refers to hexamethylpararosaniline chloride, a cationic triphenylmethane dye derived from aniline, and to commercial mixtures of triphenylmethane dyes with hexamethylpararosaniline chloride as the predominant constituent.

Gentian violet, which produces a vibrant purple color, has longstanding use as a biological and histological dye, and is a key stain in the Gram method for categorizing bacteria. In addition to such use, gentian violet has commercial and consumer applications; current commercial dye uses of gentian violet include production of inks and toners as well as coloration of papers and textiles. Applications related to gentian violet's antibacterial, antifungal, antihelminthic, antitrypanosomal, and antiviral properties include use to promote general wound healing, and in the treatment of bacterial skin infections and fungal infections, including treatment of thrush of the nipple in breastfeeding mothers and oral thrush in infants. Formulations currently available to consumers in the US include antibacterial foams and solutions of 1 - 2% gentian violet intended for topical use. Such solutions have also been adapted by consumers for cosmetic use, such as do-it-yourself hair dye.

The use of gentian violet in animal feed or as a veterinary drug in food animals is prohibited in the US. The US Food and Drug Administration (US FDA) monitors domestic and imported seafood for gentian violet residues and has issued import alerts for animal products containing gentian violet residues from a number of countries. Gentian violet has been detected in treated water effluents, seawater, and some seafood. In the US, exposure to gentian violet may result from oral and topical use of gentian violet solutions, contact with inks, commercial dyes, and biological stains, and the consumption of contaminated seafood. There is also potential for occupational exposure to gentian violet.

Human data on the carcinogenicity of gentian violet are sparse. One hospital-based retrospective study of cancer in subjects that had received transfusions of blood treated with gentian violet and a single case report of leukemia in an individual five months after coming in contact with ink containing gentian violet provide little information relevant to the chemical's carcinogenicity.

Gentian violet induced tumors at multiple sites in rats and mice in long-term carcinogenicity studies. In the two rat studies, male and female F344 rats were

exposed to gentian violet *in utero*, during lactation, and via feed post-weaning for up to 24 months, with additional interim sacrifice groups at 12 and 18 months (Littlefield et al. 1989; NCTR 1988). In the two mouse studies, male and female B6C3F1 mice were exposed to gentian violet in feed for up to 24 months (Littlefield et al. 1985; NCTR 1983). Tumor findings are as follows:

Liver tumors

- In the male F344 rats, the incidence of hepatocellular adenoma was significantly increased in the highest dose group by pairwise comparison with controls, with a significant dose-related trend. In addition, one hepatocellular carcinoma was observed in the low-dose group.
- In the male B6C3F1 mice, the incidence of hepatocellular adenoma was significantly increased in the mid- and high-dose groups by pairwise comparison with controls, with a significant dose-related trend. Hepatocellular carcinoma was significantly increased in the high-dose group by pairwise comparison with controls, with a significant dose-related trend.
- In the female B6C3F1 mice, the incidences of hepatocellular adenoma and carcinoma were both significantly increased in the mid- and high-dose groups by pairwise comparison with controls, with significant dose-related trends.

Thyroid tumors

- In the male F344 rats, the incidence of thyroid gland follicular cell adenocarcinoma was significantly increased in the low- and high-dose groups by pairwise comparison with controls, with a significant dose-related trend. The incidence of thyroid gland follicular cell adenoma or adenocarcinoma combined was significantly increased in the highest dose group by pairwise comparison with controls, with a significant dose-related trend.
- In the female F344 rats, the incidences of thyroid gland follicular cell adenocarcinoma and adenoma or adenocarcinoma combined were significantly increased in the mid- and high-dose groups by pairwise comparison with controls, with significant dose-related trends. These tumors are rare in untreated female F344 rats.

Testis and epididymis tumors

- In the male F344 rats, a dose-related increase in mesothelioma of the testis and epididymis was observed in the mid- and high-dose groups, and an increase was also seen at these dose groups in the 18-month interim sacrifice groups.

Mononuclear cell leukemia

- In the female F344 rats (18-month interim sacrifice groups), the incidence of mononuclear cell leukemia (MNCL) was significantly increased in the highest dose group by pairwise comparison with controls, with a significant dose-related trend. Although no treatment-related increase in MNCL was apparent in animals at 24 months, NCTR (1988) stated that "dosing with gentian violet was significantly associated with an earlier onset and increased mortality due to leukemia".

Clitoral gland tumors

- In the female F344 rats, a dose-related increase in clitoral gland adenoma or adenocarcinoma combined was observed in the mid- and high-dose groups.

Harderian gland tumors

- In the male B6C3F1 mice, the incidence of Harderian gland adenoma was significantly increased in the mid- and high-dose groups by pairwise comparison with controls, with a significant dose-related trend.
- In the female B6C3F1 mice, the incidence of Harderian gland adenoma was significantly increased in all three treated groups by pairwise comparison with controls, with a significant dose-related trend.

Reticulum cell sarcoma (type A), *likely histiocytic sarcoma*

- In the female B6C3F1 mice, the incidence of type A reticulum cell sarcoma (likely histiocytic sarcoma) was significantly increased in the mid- and high-dose groups by pairwise comparisons with controls, with a significant dose-related trend, in each of the following tissues:
    - Bladder
    - Ovaries
    - Uterus
    - Vagina

Metabolism of gentian violet can occur by both oxidative and reductive processes, and several of the metabolites formed are mutagenic and/or carcinogenic. Oxidative metabolites of gentian violet have been measured in *in vivo* studies of mice, rats, chickens, and microbes, and *in vitro* studies with liver microsomes isolated from mice, rats, guinea pigs, hamsters, and chickens. Reductive metabolites have been measured *in vivo* in studies of mice, rats, fish and intestinal microflora isolated from rats, mice, and

humans. Free radicals can be formed by either reductive or oxidative metabolism, with a carbon-centered free radical formed during reductive metabolism and a nitrogen-centered radical formed during oxidative metabolism. Carcinogenic metabolites include the known carcinogens formaldehyde, C.I. Basic Red 9, and Michler's ketone (the latter two are products of microbial metabolism and may be produced by intestinal microflora). Other mutagenic metabolites include pentamethylpararosaniline, N,N,N',N'- and N,N,N',N''-tetramethylpararosaniline, which are products of oxidative metabolism, and leucogentian violet and leuco-pentamethylpararosaniline, which are products of reductive metabolism.

Gentian violet has tested positive for a number of genotoxicity endpoints:

- Mutations in *Salmonella typhimurium*, *and E. coli*
- DNA damage in *B. subtilis*, *E. coli*, and mouse lymphocytes
- Binding to cell-free calf thymus DNA and synthetic polynucleotides, bacteriophage DNA, bacterial DNA, and chromosomes undergoing mitosis ("mitotic figures") in human oral mucosa tissue
- Chromosomal aberrations (CAs) in Chinese hamster ovary (CHO) cells, human HeLa cells and cultured lymphocytes, and other mammalian cells
- Chromosome breakage in CHO and human peripheral blood cells
- Gene amplification in a SV40-transformed hamster cell line

The following gentian violet metabolites have also tested positive for genotoxicity:

- Pentamethylpararosaniline chloride (both a metabolite, and a small constituent of commercial gentian violet preparations) induces mutations in *Salmonella typhimurium* (four strains), *B. subtilis, E. coli*, and bacteriophage T4D, and binds to calf thymus DNA.
- Leucogentian violet induces mutations in *Salmonella typhimurium*.
- Leuco-pentamethylpararosaniline induces mutations in *Salmonella typhimurium*.
- N,N,N',N'-tetramethylpararosaniline induces mutations in *Salmonella typhimurium* and *E. coli*.
- N,N,N',N''-tetramethylpararosaniline induces mutations in *Salmonella typhimurium* and *E. coli*.
- The carcinogenic metabolites formaldehyde, C.I. Basic Red 9, and Michler's ketone are also genotoxic.

The biological activity of gentian violet was compared to seven structurally related compounds, two of which (C.I. Basic Red 9, and Michler's ketone) are listed as carcinogens under Proposition 65.

- Common target tumor types observed between gentian violet and some of the comparison chemicals are:
    - Hepatocellular tumors (observed for three comparison chemicals)
    - Thyroid tumors (observed for one comparison chemical)
    - Harderian gland tumors (observed for one comparison chemical)
- Six of the comparison compounds have genotoxic activity.

Gentian violet may act via multiple mechanisms, including being electrophilic or forming electrophilic metabolites, genotoxicity, oxidative stress induction, and receptor-mediated effects.

- Gentian violet is a direct acting electrophile that reacts with DNA and other nucleophiles. Gentian violet also forms electrophilic metabolites, such as free nitrogen- or carbon-centered radicals formed during metabolic N-demethylation and reduction reactions.
- Gentian violet is genotoxic, as summarized above.
- Gentian violet induces oxidative stress by forming reactive oxygen species. In addition, gentian violet is active in seven ToxCast high-throughput screening (HTS) assays that have been mapped to this key characteristic of carcinogens.
- Gentian violet can modulate receptor-mediated effects, based its activity in 18 ToxCast HTS assays that have been mapped to this key characteristic of carcinogens.

2. INTRODUCTION

2.1 Identity of Gentian Violet

“Gentian violet” refers to hexamethylpararosaniline chloride, a cationic triphenylmethane dye derived from aniline, and to commercial mixtures of triphenylmethane dyes with hexamethylpararosaniline chloride as the predominant constituent. “Crystal violet” has been used interchangeably with gentian violet to refer to both hexamethylpararosaniline chloride and to commercial mixtures of triphenylmethane dyes with hexamethylpararosaniline chloride as the predominant constituent. There are additional terms used to refer specifically to gentian violet; some of the more common examples, such as “methyl violet 10B”, are given in Table 1.

The term “methyl violet” typically refers to pentamethylpararosaniline chloride, a minor constituent of the gentian violet mixture. However, “methyl violet” has occasionally been used to refer to hexamethylpararosaniline chloride and commercial mixtures of triphenylmethane dyes with hexamethylpararosaniline chloride as the predominant constituent (e.g., Kumar et al. 2011; Schaeppi 1955). For the purpose of this document, the term “methyl violet” will be used only to refer to pentamethylpararosaniline chloride.

Structures of aniline, triphenylmethane, and gentian violet are given in Figure 1. The possible identities of the various substituents (R1, R2, R3, and R4) depicted in the structure of gentian violet (Figure 1c) are indicated in the inset table.

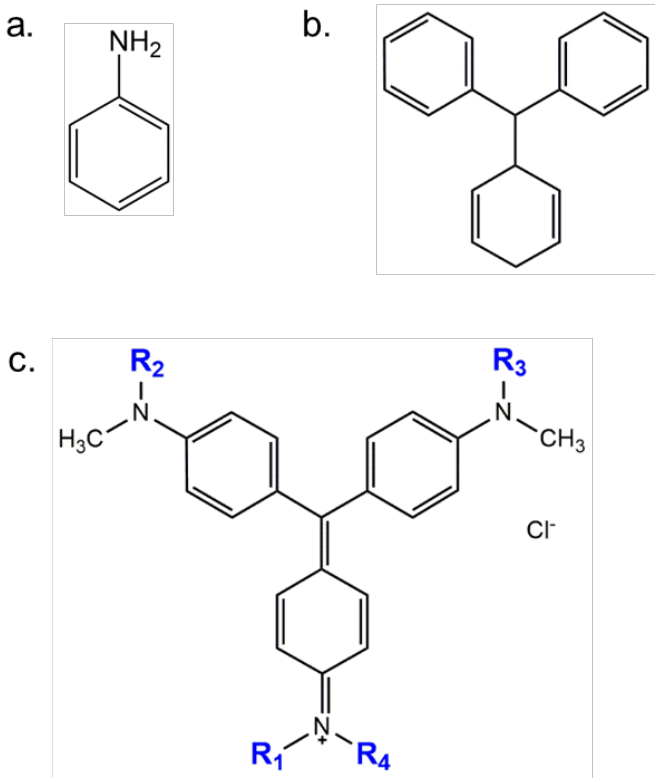


Figure 1. Structures of aniline (a), triphenylmethane (b), and gentian violet (c)

Typical Composition of Commercial Gentian Violet	Substituents				% Concentration
	R1	R2	R3	R4	
Hexamethylpararosaniline chloride *[AKA Gentian Violet; Crystal Violet]*	CH_3	CH_3	CH_3	CH_3	Typically > 96%
Pentamethylpararosaniline chloride *[AKA Methyl Violet]*	CH_3	CH_3	CH_3	H	If present, typically < 4%
N,N,N',N'-Tetramethylpararosaniline chloride	H	CH_3	CH_3	H	If present, typically < 4%
N,N,N',N''-Tetramethylpararosaniline chloride	CH_3	CH_3	H	H	
N,N',N'-Trimethylpararosaniline chloride	CH_3	H	H	H	If present, typically trace

AKA: also known as

Select chemical and physical properties are given in Table 1.

Table 1. Chemical and physical properties of gentian violet

Name	**Gentian Violet** (Hexamethylpararosaniline chloride)
IUPAC Systematic Name	[4[bis[4(dimethylamino)phenyl] methylidene]cyclohexa-2,5-dien-1-ylidene]-dimethylazanium;chloride
CAS Registry Number	548-62-9
Molecular Formula	$C_{25}H_{30}N_3Cl$
Molecular Weight	407.99 g/mol
Melting Point	215°C (decomposes)
Density	1.19 g/cm^3 at 20°C[a]
Water Solubility	4,000 mg/L at 25°C
Vapor Pressure	1.02x10^{-13} mm Hg at 25°C (estimated)
Log K_{ow} (Octanol-water)	0.51
Select Synonyms	Crystal Violet, Methyl Violet 10B, C.I. Basic Violet 3, C.I. No. 42555, Hexamethyl-p-rosaniline chloride, Methylrosanilinium chloride

[a] US Pharmacopeia (2014) (accessed on June 1, 2018)

2.2 Synthesis of Gentian Violet

Synthesis of gentian violet dates to the 19th century, with several known methods of production that result in mixtures of varying purity. The earliest reported synthesis of gentian violet is attributed to Charles Lauth, a French chemist (Gessner and Mayer 2000; Maley and Arbiser 2013). This initial synthesis was achieved through oxidation of dimethylaniline with copper salts, which resulted in formation of a mixture containing tetra-, penta-, and hexa- methylated pararosaniline chlorides (Passmore 1890). Another synthesis method reacts dimethylaniline with carbonyl dichloride, this method generates Michler's ketone, 4,4'-bis(dimethylamino)benzophenone, as an intermediate (Gessner and Mayer 2000). If the dimethylaniline precursor is pure, its reaction with carbonyl dichloride should yield hexamethylpararosaniline chloride (Harvey 1990).

Related synthesis methods that replaced carbonyl dichloride with chloropicrin or carbon tetrachloride were subsequently introduced (Cooksey 2017). Gentian violet can also be synthesized by oxidation of aniline and *p*-toluidine (Harvey 1990), or by oxidation of an intermediate leuco dye (leucogentian violet) following formation of the intermediate by condensation of formaldehyde and dimethylaniline (Gessner and Mayer 2000).

2.3 Use, Occurrence, and Exposure

Use

Gentian violet, which produces a deep, vibrant purple color, found early uses as a biological and histological dye, including as a key stain in the Gram method for categorizing bacteria and as a nuclear stain, and as a commercial dye (Berry et al. 1996; Cooksey 2017; Popescu and Doyle 1996). Gentian violet continues to be used in biological and histological stains, with the addition of novel *in vivo* biomedical stain applications (Sheng and Chen 2017; Suzuki et al. 2016). Gentian violet is also still used as a commercial dye. Applications include the coloration of paper, textiles, and related materials, and in particular elastic fibers, such as acrylic fibers (Cooksey 2017; Gessner and Mayer 2000; Thetford 2013). Gentian violet is also used to produce commercial inks for writing implements, such as indelible pencils and ballpoint pens, as well as inkjet inks and toners (Cooksey 2017; Sun et al. 2016). A review by the European Chemicals Agency (ECHA 2012) noted additional potential dye applications for gentian violet, including use in crayons, soaps, lacquers, and wood.

Therapeutic uses of gentian violet are also now relatively common; gentian violet is known to have antibacterial, antifungal, antihelminthic, antitrypanosomal, and antiviral properties, although efficacy varies (Maley and Arbiser 2013). As an antitrypanosomal agent, gentian violet has been incubated with banked blood prior to transfusion to prevent transmission of Chagas disease in endemic areas such as Latin America (Ramirez et al. 1995; Vilaseca et al. 1966). Therapeutic applications include promotion of general wound healing (Farid et al. 2011) as well as treatment of bacterial infections of the skin (Berrios and Arbiser 2011), fungal infections including thrush of the nipple in breastfeeding mothers and oral thrush in infants (Goldstein 2015) as well as vaginal yeast infections[2], burns (Arsalan Ali et al. 2013), and radiation-induced dermatitis (Bolderston et al. 2006; Maley and Arbiser 2013). Therapeutic formulations currently

[2] Prescription preparations for vaginal yeast infections were discontinued in the US in 1990; however, online sources provide instructions for "do-it-yourself" preparations. One such source is the website of a gynecologist/obstetrician, which receives several million views annually: https://drjengunter.wordpress.com/2013/10/17/how-to-use-gentian-violet-for-a-vaginal-yeast-infection/

available in the US include antibacterial foams approved by the US Food and Drug Administration (FDA) (Edwards 2016; Woo and Heil 2017) as well as solutions of 1 - 2% gentian violet, intended for topical use, that are readily available without a prescription. Such solutions have also been adapted for cosmetic use, such as do-it-yourself hair dye[3].

Although veterinary uses of gentian violet are prohibited in the US, gentian violet is used in other countries as a topical treatment of skin and eye infections in domestic animals, and for the treatment of fungal and parasitic infections in fish and other seafood in aquaculture. In some countries gentian violet is also used in animal feed to inhibit fungal growth, including mold (Kamyab et al. 2009). The FDA prohibits use of gentian violet in animal feed or as a veterinary drug in food animals (21 CFR 500.29, 21 CFR 500.30, and 21 CFR 589.1000). The FDA monitors domestic and imported seafood for gentian violet residues and has issued import alerts for animal products containing gentian violet residues from a number of countries, as these contaminated products are considered adulterated in the US (FDA 2008, 2018).

Occurrence and Exposure

Gentian violet is a contaminant in some surface waters. Gentian violet has been detected in treated effluents in China, at concentrations ranging from below 0.030 µg/L to 0.049 µg/L (Zhang et al. 2012). Gentian violet concentrations have also been reported in seawater, including water samples from aquaculture sites (0.92 µg/L) and a coastal sea (0.52 µg/L) in China (Lee et al. 2010).

Gentian violet is also found in biota as a result of intentional use and bioaccumulation from contaminated environments. To date, the FDA has detected gentian violet in concentrations up to 26.9 ng/g in imported fish (FDA 2018). Researchers have quantified gentian violet in seafood samples from Asia, including eel, fish, and shrimp, with concentrations ranging from levels below limits of detection to 168.4 ng/g (Lee et al. 2010; Lian and Wang 2013). In a German study, 35 of 45 wild eels caught downstream from municipal sewage treatment plants had detectable levels of gentian violet and its leuco metabolite up to, respectively, 0.35 ng/g and 6.7 ng/g (Schuetze et al. 2008). A recent US study of processed fish 'nuggets' from domestically raised catfish quantified gentian violet (1.1 ng/g) in 1 of 24 samples tested (Ozbay et al. 2013).

[3] Instructions for do-it-yourself hair dye are readily found online, such as https://www.wikihow.com/Temporarily-Colour-Your-Hair-Purple-at-Minimal-Cost. Also, a search with the term "gentian violet hair" on Youtube.com resulted in 828 videos on how to make hair dye at home with gentian violet, with some viewed over 408,000 times (accessed on July 12, 2018).

In the US, exposure to gentian violet may result from oral and topical use of gentian violet solutions and therapeutic preparations, contact with inks, commercial dyes, and biological stains, and the consumption of contaminated seafood. There is also potential for occupational exposure to gentian violet.

3. DATA ON CARCINOGENICITY

3.1 Carcinogenicity Studies in Humans

One hospital-based study of cancer in humans exposed to gentian violet and one case report were identified in a recent literature search conducted by OEHHA (Appendix A). The hospital-based cancer study was discussed in the following three publications, all of which were published in Portuguese and translated into English by certified translation through the UC Berkeley library service for OEHHA:

1. De Sousa et al. (1989), the report of the study, presented at a scientific conference
2. Amato Neto and Pasternak (1990), a letter to the editor with comments regarding de Souza et al. (1989)
3. Luquetti (1990), a response to Amato Neto and Pasternak (1990) by one of the authors of De Sousa et al. (1989)

As mentioned in Section 2.3, gentian violet was added to banked blood pre-transfusion to prevent Chagas disease, which is caused by the protozoan *Trypanosoma cruzi* (*T. cruzi*). Gentian violet was added to the banked blood at concentrations as high as 250 µg/mL for at least 24 hours, while the blood was stored at 4 ^{o}C (Schmunis 1999; Vilaseca et al. 1966).

De Sousa et al. (1989) interviewed 4,765 patients at the Jorge Araújo Hospital of the Cancer Association of the State of Goiás in Brazil, and asked them if they recalled getting a blood transfusion and if the blood looked purple. A sample of gentian violet-treated blood was shown to the interviewees. Out of these patients, 37 confirmed that they had received gentian violet treated blood, between 2-27 years ago. In 26 of these 37 patients, a benign or malignant neoplastic lesion was confirmed through reference to medical records. Among the 26 cases, 18 were benign and 8 were possibly malignant. The specific sites of these lesions were not reported, and risk estimates were not presented by the authors. No further information or study details were provided.

Amato Neto and Pasternak (1990) pointed out some limitations of the De Sousa et al. (1989) study, including the lack of controls, the selection bias that the interviewees were patients in a hospital that is "affiliated with an 'association of combating cancer'", the reliance on patients' recollection without verifying with the blood bank, and confounding factors such as higher iron levels and immunosuppression in blood transfusion recipients.

Professor Alejandro Luquetti, one of the original authors of (De Sousa et al. 1989), later published a response to Amato Neto and Pasternak (1990) in the form of a letter to the editor (Luquetti 1990). In the response, Luquetti stated that half of the patients treated at the hospital did not have cancer, and that this was merely a preliminary effort. On the issue of lack of controls, Luquetti stated that the cases were being compared to patients who did not receive any blood transfusions, or received red blood (as compared to purple), or who could not recall what color the blood was. However, the incidence of cancer in each of these groups was not given. He also mentioned that a central part of the project was still underway, in an attempt to match the "cases" (mostly lymphomas and leukemias) with the controls.

The case report was published in German, and describes a case of leukemia in an individual exposed to what the author called "methyl violet"[4] (Schaeppi 1955). A 57-year old man accidentally stabbed his right hand in the skin fold between two fingers with an ink pen containing gentian violet of unknown concentration. Eight weeks later, he developed swollen lymph nodes. The first swollen lymph nodes were observed at the right axilla and the right arm. Five months after the incident, the man was diagnosed with lymphocytic leukemia. The author stated that a causal link between the ink pen incident and the leukemia was assumed, although no blood work had been performed on the patient at the time of the incident.

3.2 Carcinogenicity Studies in Animals

OEHHA identified multiple carcinogenicity studies of gentian violet conducted in experimental animals. Two dietary studies in Fischer 344 (F344) rats (one in males, one in females) (Littlefield et al. 1989; NCTR 1988) and two dietary studies in B6C3F1 mice (one in males, one in females) (Littlefield et al. 1985; NCTR 1989) were conducted by the National Center for Toxicological Research (NCTR). These NCTR animal bioassays have also been reviewed and summarized by the Joint Food and Agriculture

[4] The author also noted that it was a mixture of chlorides of penta- and hexa-methylpararosanilines, so the substance in the ink pen would be what we call "gentian violet", instead of "methyl violet".

Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives, JECFA (2014).

As briefly discussed by NCTR (1983), two other sets of long-term studies of gentian violet were conducted in rats and published in the 1940s. One is Kinosita (1940) and the other is Fitzhugh (1949, an unpublished study). Kinosita (1940) administered gentian violet (referred to by the author as 4:4':4''-hexamethyltriaminotriphenylmethane) to rats orally over a period of more than 300 days, and reported findings of "gastric papilloma and a slight adenomatous proliferation in the liver". However, the publication provides no information on the doses administered, the number of rats per group, or whether the study included controls. In summarizing both the Kinosita (1940) and Fitzhugh (1949) studies, NCTR (1983) noted the significant study design issues and other limitations of these two sets of studies:

> "Two chronic studies were conducted in rats; however, no conclusions were drawn since each study suffered from several design and/or conduction deficiencies. Kinosita (1940) detected gastric papillomas and an adenomatous proliferation of hepatic tissue in rats after dosing for over 300 days. The dose level was not stated. Fitzhugh (unpublished data, FDA files, 1949) treated rats for up to two years at levels up to 1600 ppm. These inconclusive data revealed dose-related hepatic neoplastic nodules and dysplastic foci that were more severe in females."

3.2.1 Lifetime exposure studies in male and female F344 rats (Littlefield et al. 1989; NCTR 1988)

Male and female weanling F344 rats (F_0) were given gentian violet (99% hexamethylpararosaniline chloride, 1% pentamethylpararaosaniline chloride, and <<1% tetramethylpararosaniline chloride) in the feed at dose levels of 0, 100, 300, or 600 ppm for at least 80 days. While receiving dosed feed, the female rats were mated with males of the same dose level (F_0). Through communication with NCTR scientists, OEHHA confirmed that gentian violet remained in the diet of the test animals during mating, gestation, and lactation. Therefore, F_1 rats were exposed *in utero* and via lactation. Two F_1 males and two F_1 females were randomly selected from each litter and allocated to the long-term studies. The F_1 weanling rats received gentian violet in the feed at the same dose levels (0, 100, 300, or 600 ppm) as their F_0 parents. According to JECFA (2014), these dose levels were approximately equivalent to 0, 30, 80, and 160 mg/kg bw per day for male F_1 rats, and 0, 40, 100, and 200 mg/kg bw per day for female F_1 rats.

All F_1 animals received feed and drinking water *ad libitum*, for up to 24 months. The male and female studies each had 180 controls and 90 animals per gentian violet-dosed group. An additional two sets of animals with 15 rats/dose group were included in each study for interim sacrifice at 12 and 18 months.

Males

A statistically significant increase in mortality compared to the controls was observed in the 300 ppm group, starting around week 95. The average bodyweight in the 600 ppm group was statistically significantly lower than the controls, starting at about week 28, and a decrease in feed consumption was also observed in this group, as compared with controls.

Tumors observed in the male rat study are presented in Table 2. A statistically significant increase in liver hepatocellular adenomas was seen in the 600 ppm dose group, compared to controls, with a positive dose-related trend. NCTR (1988) reported that one hepatocellular carcinoma was also observed in the 100 ppm group. In the 18-month interim sacrifice groups one hepatocellular adenoma was found in the 100 ppm group. No tumors were observed at any site in the animals sacrificed at 12 months. A statistically significant increase in thyroid follicular cell adenocarcinomas was observed at both the 100 and 600 ppm dose levels by pairwise comparison with controls, with a statistically significant dose response. There was also a statistically significant increase in thyroid follicular cell adenomas or adenocarcinomas (combined) in the 600 ppm group by pairwise comparison with controls, with a positive dose-related trend. In the 18-month interim sacrifice groups one thyroid follicular cell adenoma was observed in each of the 300 and 600 ppm groups. Increases in mesotheliomas of the testis and epididymis were observed in the 300 and 600 ppm groups, and increases were also seen at these same doses in the 18-month interim sacrifice groups (NCTR 1988).

Non-neoplastic pathology findings

The authors reported almost no treatment-related non-neoplastic findings at 12- and 18-month necropsies. In animals on test for up to 24 months, several non-neoplastic pathology findings were observed in the liver, thyroid gland, spleen, and lymph nodes of gentian violet treated male rats. In the liver, a positive dose-related trend was observed for clear cell foci, eosinophilic foci, basophilic foci, mixed cell foci, cytoplasmic vacuolization, centrilobular necrosis, and liver regeneration. Specifically, clear cell foci and basophilic foci were significantly increased in the 600 ppm group, eosinophilic foci and centrilobular necrosis were increased in the 300 and 600 ppm groups, and mixed cell foci, cytoplasmic vacuolization, and liver regeneration were increased in all treated

groups. Cholesterol and triglyceride levels were increased in the 100 ppm group, compared to controls, while triglyceride levels were decreased in the 600 ppm group compared to controls. In addition, thyroid gland follicular cysts and spleen red pulp hyperplasia were increased in the 600 ppm group with a positive dose-response trend. In the lymph nodes, mesenteric reticulum cell hyperplasia was increased in the 300 and 600 ppm groups, with a positive dose-related trend.

Table 2. Tumor incidence[1, 2] in male F344 (F_1) rats exposed to gentian violet *in utero*, during lactation, and via feed post-weaning for up to 24 months (Littlefield et al. 1989 and NCTR 1988)

Tumor type	Timing of assessment	Concentration in feed (ppm)				Trend test p-value[3]
		0	100	300	600	
Hepatocellular adenoma	18 months	0/15	1/15	0/15	0/14	NS
	Up to 24 months	1/179	1/90[4]	3/88	4/89*	p<0.05
Thyroid gland follicular cell adenoma (rare)	18 months	0/15	0/15	1/15	1/15	NS
	Up to 24 months	1/163	0/84	0/74	2/79	NS
Thyroid gland follicular cell adenocarcinoma	18 months	0/15	0/15	0/14	0/13	NS
	Up to 24 months	1/163	4/84*	2/74	5/79*	p<0.05
Thyroid gland follicular cell adenoma or adenocarcinoma (combined)	18 months	0/15	0/15	1/15	1/15	NS
	Up to 24 months	2/163	4/84	2/74	7/79**[5]	p<0.01
Testis and epididymis mesothelioma[6]	18 months	0	0	13%	13%	NA
	Up to 24 months	3%	2%	6%	9%	NA

[1] Tumor incidence is expressed as the number of rats with the specified neoplastic lesion divided by the number of rats examined at the site.

[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

[3] Exact trend test conducted by OEHHA. NS, not significant ($p \geq 0.05$). NA, not applicable.

[4] In addition, another animal in this group had a hepatocellular carcinoma.

[5] Littlefield et al. (1989) reported as 7/78.

[6] Incidences reported only as percentages.

Females

A statistically significant increase in mortality compared to the controls was observed in the 300 and 600 ppm females after the first year. NCTR (1988) attributed the increased mortality in the mid- and high-dose groups to mononuclear cell leukemia (MNCL), which occurred earlier and was more lethal in gentian violet-treated animals than in controls. The average bodyweight in the 600 ppm group was significantly lower than that of controls and the difference was more apparent in the second year. At the end of the study, the average bodyweight in the 300 ppm group was also significantly lower than that of the controls. No differences in feed consumption were observed between control and treated female rats.

Tumors observed in the female rat study are presented in Table 3. Statistically significant increases in thyroid follicular cell adenocarcinomas, and adenomas or adenocarcinomas combined were seen in the 300 and 600 ppm groups by pairwise comparison, with positive dose-related trends. One thyroid follicular cell adenocarcinoma was observed in 100 ppm females in the 18-month interim sacrifice groups. Thyroid follicular cell adenomas and (adeno)carcinomas are rare in untreated female F344 rats in NCI and NTP two-year feeding studies (Goodman et al. 1979; Haseman et al. 1985). No tumors were observed at any site in the animals sacrificed at 12 months. A statistically significant increase in MNCL was observed at 600 ppm in the 18-month interim sacrifice groups, with a significant dose-related trend. Although no treatment-related increase in MNCL was apparent in animals on test for up to 24 months, NCTR (1988) reported that “dosing with gentian violet was significantly associated with an earlier onset and increased mortality due to leukemia” in this study. Increases in clitoral gland adenoma and adenocarcinoma combined were also observed in the 300 and 600 ppm groups, with NCTR (1988) noting that the increases were “suggestive of a treatment-related trend”.

Non-neoplastic pathology findings

The authors reported almost no treatment-related non-neoplastic findings at 12- and 18-month necropsies. In animals on test for up to 24 months, significant increases of non-neoplastic findings were seen in the liver and bone marrow. In the liver, mixed cell foci, liver regeneration, and centrilobular fatty change were significantly increased in all treated female rats compared to controls, with positive dose-related trends. Eosinophilic foci and bile duct hyperplasia were increased in both 300 and 600 ppm groups with dose-related trends. There was a statistically significant increase of centrilobular necrosis as well as hematopoietic cell proliferation in the 600 ppm group, with a dose related trend. An increase in liver clear cell foci was seen in the 300 ppm

group, but not in other treated groups. Cholesterol levels were increased in the 600 ppm group compared to controls, and triglyceride levels were decreased in the 300 and 600 ppm groups, compared to controls. In addition, bone marrow hyperplasia was increased in the 300 and 600 ppm groups, with a dose-related trend.

Table 3. Tumor incidence[1, 2] in female F344 (F_1) rats exposed to gentian violet *in utero*, during lactation, and via feed post-weaning for up to 24 months (Littlefield et al. 1989 and NCTR 1988)

Tumor type	Timing of assessment	Concentration in feed (ppm)				Trend test p-value[3]
		0	100	300	600	
Thyroid gland follicular cell adenoma (rare)	18 months	0/15	0/11	0/10	0/14	NS
	Up to 24 months	1/159	2/83	3/76	3/77	NS
Thyroid gland follicular cell adenocarcinoma (rare)	18 months	0/15	1/11	0/10	0/14	NS
	Up to 24 months	1/159	1/83	4/76*	6/77**	p<0.01
Thyroid gland follicular cell adenoma or adenocarcinoma (rare) (combined)	18 months	0/15	1/11	0/10	0/14	NS
	Up to 24 months	2/159	3/83	7/76**	9/77***	p<0.001
Mononuclear cell leukemia	18 months	0/15	2/11	2/10	6/14**	p<0.01
	Up to 24 months	77/171	38/90	45/87	40/87	NS
Clitoral gland adenoma or adenocarcinoma (combined)[4]	Up to 24 months	12%	6%	18%	33%	NA

[1] Tumor incidence is expressed as the number of rats with the specified neoplastic lesion divided by the number of rats examined at the site.

[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

[3] Exact trend test conducted by OEHHA. NS, not significant ($p \geq 0.05$). NA, not applicable.

[4] Incidences reported only as percentages.

3.2.2 24-month feed studies in male and female B6C3F1 mice (Littlefield et al. 1985; NCTR 1983)

Male and female B6C3F1 (C57BL/6 x C3H) mice 4-5 weeks of age were given gentian violet (99% hexamethylpararosaniline chloride, 1% pentamethylpararosaniline chloride, and <<1% tetramethylpararosaniline chloride) in the feed at dose levels of 0, 100, 300 and 600 ppm for up to 24 months. According to JECFA (2014), the dose levels were approximately equivalent to 0, 10.7-14.3, 32.1-35.7 and 64.3 mg/kg bw per day for male mice, and 0, 14.3, 35.7-39.3 and 71.4 mg/kg bw per day for female mice. All animals received feed and drinking water *ad libitum*, for up to 24 months. The male and female studies each had 180 controls and 90 animals per gentian violet-dosed group. Additionally, two sets of animals with 15 mice/dose group were included in each study for interim sacrifice at 12 and 18 months.

Males

The mortality rates for all groups of male mice were very low until approximately 500 days, after which there was a significant dose-related trend in increased mortality. There were no differences in bodyweight between the controls and any of the gentian violet-treated groups.

Tumors observed in the male mouse study are presented in Table 4. There were statistically significant increases in hepatocellular adenomas at 300 and 600 ppm, and in hepatocellular carcinomas at 600 ppm, with positive dose-related trends. Lung metastases of liver tumors were observed in some mice, and occurred in 3/93 animals in the 600 ppm group compared to 3/183 in controls. No clear treatment-related increase in tumors at any site was observed at either the 12- or 18-month interim sacrifice. In addition, the incidence of Harderian gland adenomas was significantly increased in the 300 and 600 ppm groups, with a dose-related trend.

Non-neoplastic pathology findings

No treatment-related non-neoplastic pathology findings were reported by NCTR (1983) or Littlefield *et al.* (1985).

Table 4. Tumor incidence[1,2] in male B6C3F1 mice fed gentian violet in the diet for up to 24 months (Littlefield et al. 1985 and NCTR 1983)

Tumor type	Timing of assessment	Concentration in feed (ppm)				Trend test p-value
		0	100	300	600	
Hepatocellular adenoma	12 months	0/48	2/24	0/24	0/24	NS
	18 months	3/48	0/24	2/24	2/22	NS
	Up to 24 months	17/183	14/92	20/93**	37/93***	p<0.0001
Hepatocellular carcinoma	12 months	0/47	0/24	0/24	0/24	NS
	18 months	5/48	1/24	2/24	2/22	NS
	Up to 24 months	27/183	15/92	17/93	33/93***	p<0.0001
Harderian gland adenoma	12 months	1/46	0/24	0/24	0/24	NS
	18 months	2/47	2/24	2/23	0/21	NS
	Up to 24 months	7/187	7/92	10/94*	9/89*	p<0.05

[1] Tumor incidence is expressed as the number of rats with the specified neoplastic lesion divided by the number of rats examined at the site. Incidence of hepatocellular adenomas and carcinomas combined was not reported.
[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
[3] Exact trend test conducted by OEHHA. NS, not significant ($p \geq 0.05$).

Females

The mortality rate for all groups of female mice was very low until approximately 560 days, after which statistically significant increases in mortality were observed in all treated groups, compared to controls. There were no differences in bodyweight between the controls and any of the gentian violet-treated groups.

Tumors observed in the female mouse study are presented in Table 5. The incidences of hepatocellular adenomas and hepatocellular carcinomas were significantly increased by pairwise comparison with controls ($p<0.001$) in the 300 ppm and 600 ppm groups, with positive dose-related trends. Lung metastases of liver tumors were observed in some mice, with none in controls, one instance in the 100 ppm group, three in the 300 ppm group, and 13 in the 600 ppm group. In the 18-month interim sacrifice groups, hepatocellular adenomas were significantly increased in the 600 ppm group by pairwise comparison with controls, and positive dose-response trends for increases in hepatocellular adenomas and carcinomas were observed. A significant increase of Harderian gland adenomas was observed in each of the treated groups by pairwise comparison with controls, with a significant dose-response trend. In addition, statistically significant increases in the incidence of neoplasms classified by NCTR as "type A reticulum cell sarcomas" of the bladder, ovaries, uterus, and vagina occurred in the 300 and 600 ppm groups by pairwise comparison with controls, with positive dose-response trends. Reticulum cell sarcoma (Type A) is an older term for neoplasms that most likely would be classified now as "histiocytic sarcoma" (Frith et al. 1993). However, the lack of tissue slides/blocks available from this study makes it unfeasible for NCTR pathologists to confirm a new designation (email communication with NCTR).

Table 5. Tumor incidence[1,2] in female B6C3F1 mice fed gentian violet in the diet for up to 24 months (Littlefield et al. 1985 and NCTR 1983)

Tumor type	Timing of assessment	Concentration in feed (ppm)				Trend test p-value[3]
		0	100	300	600	
Hepatocellular adenoma	12 months	0/48	0/24	0/24	0/24	NS
	18 months	3/47	0/22	3/24	8/24**	p<0.001
	Up to 24 months	8/185	8/93	36/93***	20/95***	p<0.0001
Hepatocellular carcinoma	12 months	0/48	0/24	0/24	0/24	NS
	18 months	1/47	0/22	1/24	3/24	p<0.05
	Up to 24 months	7/185	5/93	30/93***	73/95***	p<0.0001
Harderian gland adenoma	12 months	2/48	0/24	1/24	0/24	NS
	18 months	2/46	2/21	3/23	1/23	NS
	Up to 24 months	8/186	11/93*	18/89***	15/94**	p<0.001
Reticulum cell sarcoma (type A)[4], Bladder	12 months	0/48	0/23	0/24	0/24	NS
	18 months	0/47	1/22	1/24	0/23	NS
	Up to 24 months	0/188	2/92	3/89*	5/91**	p<0.01
Reticulum cell sarcoma (type A)[4], Ovaries	12 months	0/47	0/23	0/22	0/24	NS
	18 months	0/45	0/21	0/22	0/21	NS
	Up to 24 months	0/178	1/90	3/89*	5/89**	p<0.01
Reticulum cell sarcoma (type A)[4], Uterus	12 months	0/47	0/23	0/24	0/24	NS
	18 months	0/47	0/22	1/24	1/24	NS
	Up to 24 months	0/188	2/95	6/90**	12/93***	p<0.0001
Reticulum cell sarcoma (type A)[4], Vagina	12 months	0/45	1/23	0/24	0/23	NS
	18 months	0/46	0/22	1/23	0/22	NS
	Up to 24 months	1/182	1/90	4/88*	8/87***	p<0.001

[1] Tumor incidence is expressed as the number of rats with the specified neoplastic lesion divided by the number of rats examined at the site. Incidence of hepatocellular adenomas and carcinomas combined was not reported.

[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

[3] Exact trend test conducted by OEHHA. NS, not significant ($p \geq 0.05$).

[4] Likely histiocytic sarcoma

Non-neoplastic pathology findings

A significant increase of spleen erythropoiesis was observed in each of the treated groups (except for a borderline increase at 100 ppm), compared to controls, with a positive dose-response trend (NCTR, 1983).

3.3 Other Relevant Data

3.3.1 Pharmacokinetics and metabolism

Information for the absorption, distribution, metabolism and elimination of gentian violet stems from studies in animals, microbes, and cell free systems. *In vivo* studies include studies in rats, mice, chickens (McDonald and Cerniglia 1984; McDonald et al. 1984a; NCTR 1985, 1989), fish (Chan et al. 2012; Thompson et al. 1999), and studies in the protozoan parasite, *T. cruzi* (Docampo et al. 1983). Several *in vitro* studies include experiments with microsomes from rats, mice, hamsters, chickens, and guinea pigs (Harrelson and Mason 1982; McDonald and Cerniglia 1984; McDonald et al. 1984b), and a study using horseradish peroxidase (HRP) as the metabolic activation system (Gadelha et al. 1992). In addition, several studies examined aerobic and anaerobic metabolism by various fungi and bacteria (Bumpus and Brock 1988; Chen et al. 2007; Kumar et al. 2011; Yatome et al. 1991; Yatome et al. 1993), including the metabolites formed by human and animal microflora (McDonald and Cerniglia 1984).

Absorption

Absorption studies of gentian violet in mammals are limited but indicate rapid absorption of gentian violet upon gavage (within 2 hours) of a single dose in male and female rats (McDonald et al. 1984a; NCTR 1989). Absorption of gentian violet is thought to be greater compared to other triphenylmethane dyes based on greater urinary and biliary excretion for gentian violet compared to other triphenylmethane dyes (see ‘Excretion’ below) (NCTR 1989). NCTR (1989) reasons that the greater absorption of gentian violet may be based on its smaller molecular weight compared to other triphenylmethane dyes, as well as its neutral charge in the intestinal environment which may increase its permeability. Absorption in rats following oral administration can be indirectly estimated to be less than 10 percent, based on measures from urinary and biliary excretion experiments; absorption is estimated to be slightly higher in mice (McDonald et al. 1984a).

In chickens, gentian violet is also rapidly absorbed from the diet, reaching peak levels in blood during the first hour post administration, which rapidly decline within 4 hours (Olentine et al. 1980). Half-lives of gentian violet in blood ranged from 1.43 hours for

male to 1.68 hours for female chickens. No gentian violet residue was found in blood samples taken after 48 hours (Olentine et al. 1980). Studies in fish also indicate rapid absorption of gentian violet from the water column (1-2 hours) by fish (Chan et al. 2012; Thompson et al. 1999).

Distribution

Rodents

Upon absorption following a single gavage dose to male and female F344 rats, [phenyl-U-^{14}C] gentian violet was distributed to the kidney, liver, gonads, and fatty tissues, with the kidney and liver exhibiting the highest levels (McDonald et al. 1984a; NCTR 1989). Radioactivity was found in all tissues examined two hours post-administration and reached a peak at 4 hours in the kidney and liver. Depletion half-lives for liver and kidney were 14.5 and 14.4 hours in male and 17 and 18.3 hours in female rats. Levels in fatty tissue accumulated slowly and reached a plateau at 24 hours. A depletion rate of residue from fatty tissue could not be determined.

In multi-dose experiments male and female rats and mice were gavaged with [phenyl-U-^{14}C] gentian violet twice per day for seven days and animals were sacrificed 2 hours after the last dose (McDonald et al. 1984a; NCTR 1989). The highest residue levels (14.8 – 24 ppm) were present in the fatty tissue of female rats and male and female mice; the level in the fatty tissue of male rats was 3.2 ppm. Muscle tissues had low residue levels (0.1-1.3 ppm) in both species and sexes. Rat liver residues were approximately 4 ppm, whereas mouse livers had levels as high as 17.8 ppm in males and 10.7 ppm in females. The tissue residues included parent material (gentian violet), demethylated (penta- and tetra-methylpararosaniline) and reduced (leucogentian violet, leuco-pentamethylpararosaniline) metabolites, with fatty tissue containing the highest concentration of reduced metabolites. The metabolites were variously distributed among the tissues, sexes, and species. The parent compound ranged from 19 and 29 ppb in male rat kidney and liver, respectively, to 60 and 122 ppb in female rat kidney and liver, respectively. Adipose tissues had high levels of leucogentian violet and leuco-pentamethylpararosaniline ranging from 1813 ppb in male rats to 7043 ppb in female rats; levels for mice were between these levels.

Chickens

A 7-day multi-dose experiment was carried out with male and female Cornish-White Rock broiler chickens where [phenyl-U-^{14}C] gentian violet was administered via capsules three times per day, and animals were sacrificed 6, 24, 48, 120, and 240 hours after the last dose (NCTR 1985). Depletion of radioactive labeled gentian violet was determined in seven edible tissues, i.e., the liver, kidney, gizzard, breast, thigh, heart, and skin. Residues were detected in all tissues, with the highest levels reported in the liver of both male and female chickens. The residues included parent compound

(gentian violet), penta- and the two tetra-methylpararosaniline isomers. These compounds were only detected at the 6-hour sampling point and not at later times; the authors note that unidentified insoluble tissue residues represented a substantial portion of the total residue in most tissues at all sampling times. Depletion kinetics were bi-phasic and consistent between males and females. Half-lives for phase 1 depletion in male chickens ranged from 2.7 hours in liver to over 6 hours in kidney and muscle tissues; half-life for liver depletion in female chickens was 38.2 hours. The authors attributed the longer half-lives for phase 1 in females to fewer available data points during that phase. Phase 2 depletion of the liver had longer half-lives for both sexes, with 215 hours for males and 153 hours for females.

In another diet study in Hubbard x Hubbard broiler breeder chickens, radioactive gentian violet or metabolites were also present in edible tissues, with the highest levels reported in kidneys 8 hours post administration (Olentine et al. 1980). Radioactivity was highest in liver and kidney; levels declined quickly in the first 24 hours in all tissues, but persisted at a detectable level in the kidney until 432 hours post administration. Half-lives for kidney residues were 105 hours in males versus 10.4 hours in females. The authors had no explanation for this difference. Gentian violet and its metabolites did not accumulate in the fatty tissue; however, radioactivity was found in eggs up to 456 hours post-administration.

Fish

In fish, gentian violet was rapidly metabolized to leucogentian violet. While the parent compound was not detected in muscle tissue by 14 days post-dosing, leucogentian violet was detected in muscle tissue up to day 79 in Channel catfish and day 91 in Atlantic salmon (Chan et al. 2012; Thompson et al. 1999).

Metabolism

Metabolism of gentian violet can occur by both oxidation and reduction, and either pathway can lead to the production of free radicals as well as mutagenic and/or carcinogenic metabolites (See Figure 2 below). Oxidative metabolism has been observed *in vivo* in mice, rats, and chickens, and *in vitro* with liver microsomes from both sexes of four strains of mice, three strains of rats, guinea pigs, hamsters, and chickens (McDonald et al. 1984a; McDonald et al. 1984b; NCTR 1985, 1989) as well as during microbial biotransformation of gentian violet (Bumpus and Brock 1988; Chen et al. 2007; Kumar et al. 2011). Reductive metabolism of gentian violet occurs in rodents and fish, and has also been observed in incubations of gentian violet with cell suspensions from the intestinal microflora from rats, mice, and humans, and in microbial biodegradation studies (Chan et al. 2012; McDonald and Cerniglia 1984; NCTR 1985, 1989; Thompson et al. 1999; Yatome et al. 1991; Yatome et al. 1993). Gentian violet can undergo single electron reductions and oxidations, leading to the formation of

carbon- or nitrogen- centered free radicals, respectively. Single-electron reactions have been observed with whole cell and homogenate preparations of *T. cruzi*, with rat microsomes, during light exposure of gentian violet, and in enzymatic reactions with HRP (Docampo et al. 1983; Gadelha et al. 1992; Harrelson and Mason 1982; Leaver 1972). Further details of oxidative and reductive metabolism are described below.

Oxidative metabolism involves the demethylation of gentian violet via the sequential removal of a single methyl group at each step, resulting in the formation of pairs of isomers. These isomers are distinguished from each other by the position of where the methyl group has been removed (from the same site or from a different site of the gentian violet molecule). Demethylation yields penta-, tetra-, tri-, di-, mono- and non-methylated metabolites. The non-methylated metabolite, pararosaniline (C.I. Basic Red 9), is a known carcinogen. While complete demethylation of gentian violet (i.e., C.I. Basic Red 9) has not been assessed in animal models, it has been observed during microbial degradation. The partially demethylated metabolites pentamethylpararosaniline, and two tetramethylpararosaniline isomers, N,N,N',N'- and N,N,N',N''-tetramethylpararosaniline, all of which are positive in bacterial mutagenicity tests (Aidoo et al. 1990; Hass et al. 1986), have been identified in various tissues *in vivo* in rodents and chicken as well as *in vitro* (McDonald et al. 1984b; NCTR 1985, 1989). During demethylation reactions, formaldehyde, another known carcinogen, is formed at each demethylation step (see Figure 2) (Docampo et al. 1983; Gadelha et al. 1992). Formaldehyde production increased *in vitro* with increasing amounts of HRP, and the reaction was inhibited with reduced glutathione (GSH) and ascorbate.

Reductive metabolism of gentian violet leads to the formation of leucogentian violet, leuco-pentamethylpararosaniline, and other leuco- metabolites, possibly via the formation of a carbon-centered free radical (Docampo and Moreno 1990). Leucogentian violet, leuco-penta- and possibly leuco-tetra-methylpararosaniline have been identified in tissues of rats and mice. Leucogentian violet is the main metabolite found in the muscle tissue of fish and in the fatty tissue of rats and mice (Chan et al. 2012; NCTR 1989; Thompson et al. 1999). Leucogentian violet is also the major metabolite formed by microflora isolated from the feces or intestine from humans, rats and chickens (McDonald and Cerniglia 1984). Both leucogentian violet and leuco-pentamethylpararosaniline were mutagenic when tested in bacterial mutagenicity assays (Hass et al. 1986).

Single electron reactions and free radical formation

Gentian violet can be photochemically or electrochemically reduced or oxidized in one-electron reactions, leading to the formation of carbon- or nitrogen-centered free radicals, respectively (Docampo et al. 1983; Gadelha et al. 1992; Harrelson and Mason 1982; Leaver 1972; Reszka et al. 1986). Under anaerobic conditions, photoreduction of

gentian violet with light of greater than 500 nm wavelength led to the formation of a free carbon-centered tri(p-dimethylaminophenyl)methyl radical (Leaver 1972; Reszka et al. 1986). No such radical could be detected under aerobic conditions, but gentian violet converted oxygen to superoxide anion and hydrogen peroxide under aerobic conditions and visible light, and the reaction was enhanced by the addition of NAD(P)H (Reszka et al. 1986). A carbon centered free radical was also observed during incubation of gentian violet with rat hepatic microsomes supplemented with NADPH and under a nitrogen atmosphere, i.e., anaerobic conditions (Harrelson and Mason 1982). Radical formation was inhibited with carbon monoxide, suggesting the involvement of cytochrome P450. Similarly, radical formation also occurred when intact cells or homogenates of trypomastigotes or epimastigotes (infectious stages of *T. cruzi*) were incubated with gentian violet under a nitrogen atmosphere (Docampo et al. 1983). Light enhanced the trypanocidal action of gentian violet.

A nitrogen-centered free radical was formed when gentian violet was oxidized by HRP, a reaction that has been observed with other N-methyl substituted aromatic amines. This reaction also resulted in the formation of formaldehyde (Gadelha et al. 1992; Van der Zee et al. 1989). It is possible that the nitrogen radical is further metabolized to pentamethylpararosaniline.

Additional Pathways

In some microbes, gentian violet can be metabolized to 4,4-bis(dimethylamino)benzophenone (Michler's ketone) and N,N-dimethylaminophenol (Chen et al. 2008; Yatome et al. 1991; Yatome et al. 1993). Michler's ketone can be further metabolized to 4-hydroxybenzaldehyde and N,N-dimethylaminophenol by some microorganisms (Chen et al. 2008). Michler's ketone is a known carcinogen listed under Proposition 65 (NCI 1979; Proposition 65 List).

Gentian violet preparations used in metabolism studies

As discussed in Section 2.1 (Identity of gentian violet), gentian violet refers to hexamethylpararosaniline chloride, and to commercial mixtures of triphenylmethane dyes typically containing > 96% hexamethylpararosaniline chloride. Other constituents of the commercial mixtures typically consist of < 4% penta- and tetra-methylpararosaniline, and, if present, trace amounts of trimethylpararosaniline. Gentian violet preparations may also contain trace amounts of unreacted Michler's ketone. Both commercial grade gentian violet as well as more purified gentian violet (higher content of hexamethylpararosaniline) have been used in metabolism studies. Hence, the question arises as to whether measurements of penta- or tetra-methylpararosaniline or Michler's ketone in metabolism studies may simply reflect the presence of these compounds in the starting material. However, evidence from multiple studies indicates

that penta- and tetra-methylpararosaniline and Michler's ketone are indeed formed from gentian violet preparations via enzymatic action and thus are metabolites. Active demethylation of gentian violet is supported by experiments that demonstrate the linear decrease of the parent compound and the simultaneous formation and increase in demethylated metabolites over time (Bumpus and Brock 1988; Chen et al. 2007; Kumar et al. 2011). In addition, incubation of gentian violet with rat microsomes, but not heat-killed microsomes or extracts without microsomes, leads to the formation of demethylated products from the parent compound. Furthermore, stepwise demethylation of gentian violet was also demonstrated via the incubation of pentamethylpararosaniline with rat microsomes, which led to the disappearance of substrate and appearance of tetramethylpararosaniline (McDonald et al. 1984b). In biodegradation studies that reported Michler's ketone as a metabolite, the authors purified commercial gentian violet prior to its use (Yatome et al. 1991; 1993). In conclusion, while some unmetabolized substrate may account for a portion of the metabolites identified, these findings indicate that enzymatic demethylation of gentian violet and metabolism to Michler's ketone takes place.

Excretion

Studies in rats and mice indicate that gentian violet is excreted in the feces, with some excretion via the urine also occurring (NCTR, 1989; McDonald et al. 1984a). In male and female F344 rats, fecal excretion of the administered dose amounted to 72.9% and 63.8%, respectively; urinary excretion amounted to about 2.2% of the administered dose in both sexes (McDonald et al. 1984a). Bile duct cannulation studies conducted in female rats reported that 5.7% to 6.4% of the administered dose of gentian violet was excreted in the bile within 28 hours (McDonald et al. 1984a; NCTR 1989). In male mice, urinary excretion was about 5.9 percent of the administered and it was 8.1 percent of the administered dose in female mice (McDonald et al. 1984a; NCTR 1989).

The parent compound, gentian violet, was present in the highest concentration in the feces of female rats and mice compared to other metabolites found. In addition to the parent compound, the gentian violet metabolites pentamethylpararosaniline, the two isomers of tetramethylpararosaniline, leucogentian violet, and leuco-pentamethylpararosaniline were detected in the feces of both sexes of rats and mice (NCTR 1989).

Summary

Upon absorption, gentian violet is distributed throughout the body, initially concentrating in the liver and kidney, then accumulating in fatty tissues. Its absorption is greater compared to other triphenylmethane dyes, possibly based on a smaller molecular size and neutral charge and greater uptake in the intestine. Clearance of gentian violet

residues is biphasic. In fish, gentian violet residues can remain in the muscle tissue for up to three months. Gentian violet residues have been detected in chicken eggs up to 19 days after treatment. Excretion of gentian violet occurs primarily via feces and with some excretion occurring via urine.

Metabolism of gentian violet can be oxidative or reductive, and several of the metabolites formed are mutagenic and/or carcinogenic, and include the known carcinogens formaldehyde, C.I. Basic Red 9, and Michler's ketone (the latter two formed by microbial metabolism). Free radicals can be formed by either reductive or oxidative metabolism, with a carbon-centered free radical formed during reductive metabolism and a nitrogen-centered radical formed during oxidative metabolism (Docampo et al. 1983; Gadelha et al. 1992; Harrelson and Mason 1982). While no studies on the human metabolism of gentian violet have been identified, one study with human fecal microflora demonstrated that gentian violet can be reduced to leucogentian violet, thus indicating that reductive metabolism and possibly radical formation may occur in humans (McDonald and Cerniglia 1984).

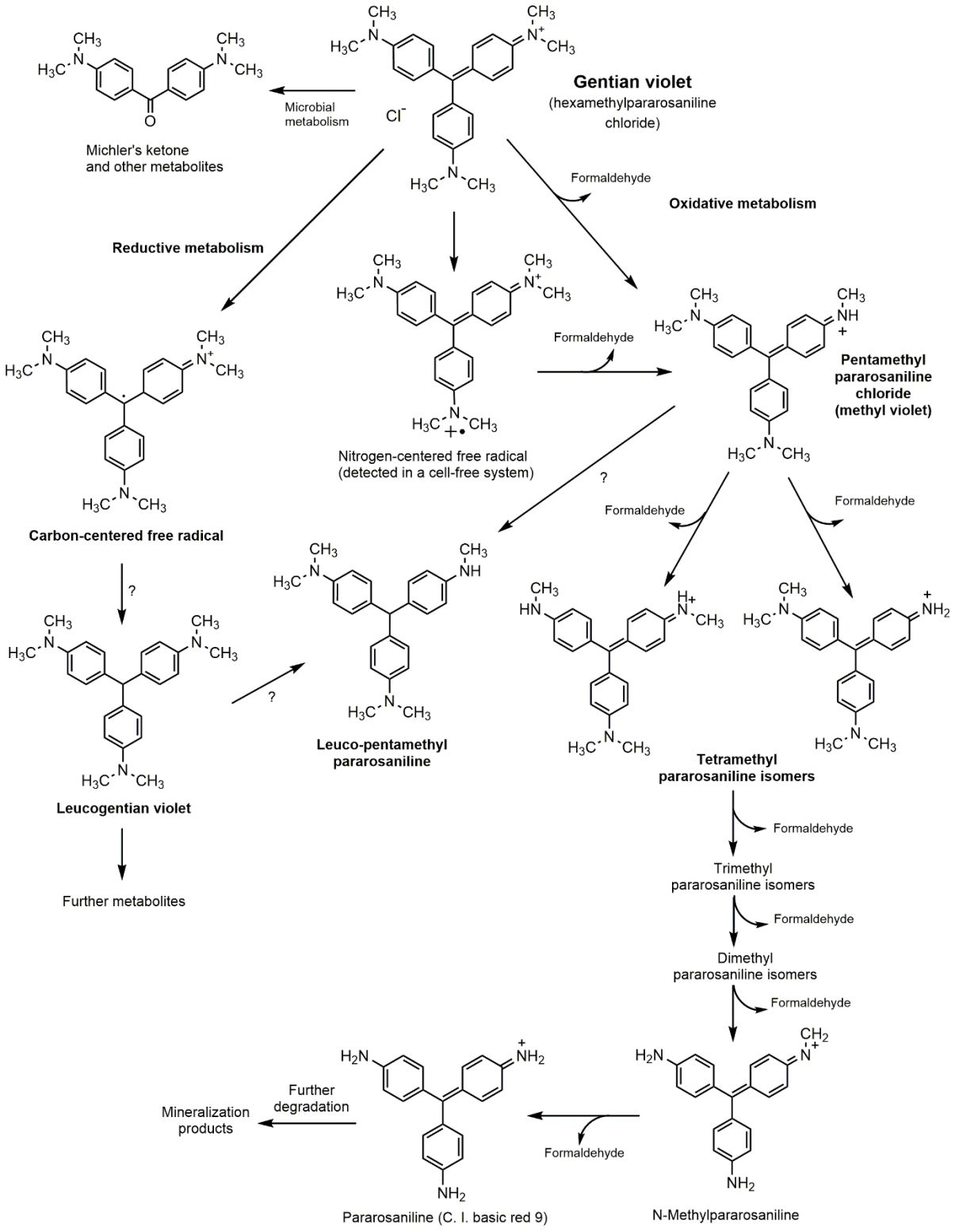


Figure 2. Proposed metabolism of gentian violet

(Modified based on Docampo and Moreno 1990; Chen et al. 2007; 2008; Yatome et al. 1991; 1993; Gadelha et al 1992; Kumar et al. 2011). Gentian violet refers to hexamethylparaosaniline chloride. Commercial mixtures of gentian violet may contain <4% pentamethylpararosaniline chloride and tetramethylpararosaniliine chloride isomers, and trace levels of trimethylpararosaniline chloride. Chemical names in **bold** indicate detection in mammalian systems.

3.3.2 Genotoxicity

The genotoxicity of gentian violet has been reviewed and summarized by JECFA (2014). In summarizing gentian violet's genotoxicity data, JECFA stated the following:

> "The data show that gentian violet binds to DNA, and this, together with the cellular toxicity of gentian violet, complicates both the testing of gentian violet in vitro and the interpretation of the results. The results are somewhat varied in *Salmonella typhimurium*, with positive responses in some strains but not in others. Gentian violet was clastogenic in vitro and positive in indicator tests for DNA damage. There are few in vivo tests on gentian violet. A single in vivo test for clastogenicity (mouse bone marrow assay) showed no evidence of clastogenic activity, but the Committee noted that the gentian violet was given via the drinking-water at lower doses (4 and 8 mg/kg bw per day) than those used in the mouse cancer bioassay (ranging from 10 to 70 mg/kg bw per day). Similarly, the other in vivo test on DNA damage in mouse lymphocytes using single intravenous doses up to 6 mg/kg bw showed no effect. The committee concluded that, overall, the data show that gentian violet is genotoxic.
>
> In view of the carcinogenicity of gentian violet in the mouse and rat and evidence showing genotoxicity in a number of tests, the Committee concluded that gentian violet should be considered a carcinogen acting by a genotoxic mode of action."

OEHHA reviewed JECFA's evaluation of the genotoxicity studies, and has included the relevant sections of JECFA (2014) here, as Appendix B.

3.3.2.1 Additional studies on the genotoxicity of gentian violet

Several additional publications on the genotoxicity of gentian violet that were not included in the JECFA (2014) review were identified in the literature search conducted by OEHHA. The findings from these additional studies are reported in the table below (Table 6). Overall, these additional studies showed positive results for bacteria mutagenicity (e.g., *Salmonella* TA 98, TA100; *E. coli* WP2), negative results in one sex-linked recessive lethal test in *Drosophila*, and positive results for DNA binding.

Table 6. Additional genotoxicity studies on gentian violet identified by OEHHA that were not included in the review by JECFA (2014)

Assay endpoint	Test system	Test article[1]	Concentration	Results	Reference
Mutagenicity	*Salmonella typhimurium* TA1535, TA1536, TA1537, and TA1538	Crystal violet	Not reported	– without S9	Sugimura et al. (1976)
Mutagenicity (Ames II assay for base pair mutations)	*Salmonella typhimurium* strains TA7001-7006	Crystal violet	0.96-960 µg/ml	–	Kamber et al. (2009)
Mutagenicity and DNA damage	Bacteria, including *B. subtilis* H17A and M45T, *Escherichia coli* WP2, and *Salmonella typhimurium* TA98 and TA100	Crystal violet	2 mg for *B. subtilis* rec assay; 0.08 mg for *E. coli* mutation assay; 16 µg/plate for *Salmonella* Ames test	+	Fujita et al. (1976), as cited by TOXLINE[2] and Fujita (1977), as cited by Littlefield et al. (1989) and Aidoo et al. (1990)
Sex-linked recessive lethal mutation	*Drosophila Melanogaster*	Crystal violet	Not reported	–	Clark (1953)
DNA binding	Calf thymus DNA (cell-free)	Crystal violet	10 µM	+	Lewis and Indig (2002)[3]
DNA binding	Calf thymus DNA (cell-free) and synthetic polynucleotides poly(rA)·poly(dT)	Crystal violet	Not reported	+[4]	Muller and Gautier (1975)
DNA binding	Calf thymus DNA (cell-free) and bacteriophage PM2 DNA (cell-free)	Crystal violet	Not clearly reported	+	Wakelin et al. (1981)

Assay endpoint	Test system	Test article[1]	Concentration	Results	Reference
DNA binding	Bacterial DNA (cell-free) from *C. perfringens, B. subtilis, E. coli,* and *M. luteus*	Crystal violet	Not reported	+	Muller and Gautier (1975)
Binding to mitotic figures [5]	Human oral mucosa tissue specimen (normal, epithelial dysplasia, or squamous cell carcinoma)	Crystal violet and nuclear fast red stain	1%	+	Motiwale et al. (2016)

[1] As named by the study authors. As discussed in Section 2.1, crystal violet is a synonym for gentian violet.
[2] This study is in Japanese. The study results are available as a permanent record from TOXLINE at https://toxnet.nlm.nih.gov/cgi-bin/sis/search2/r?dbs+toxline:@term+@DOCNO+EMICBACK/30607
[3] This study has some discussion on the oxidative damage induced by the photolysis of the non-covalent crystal violet-DNA complex.
[4] The affinity of crystal violet for the calf thymus DNA was 10-fold higher than for the synthetic mixed ribo·deoxyribo nucleotide polymers.
[5] The authors defined "mitotic figures" as "various chromosomal arrangements in cells undergoing division".

Besides genotoxicity studies, several studies have shown that gentian violet can be metabolized through single electron reduction to form carbon- or nitrogen-centered free-radicals (Docampo et al. 1983; Gadelha et al. 1992; Harrelson and Mason 1982; Lewis and Indig 2002). Docampo et al. (1983) also found that light enhanced the formation of the free radicals, which were thought to be responsible for gentian violet's toxicity towards the parasite *T. cruzi*.

3.3.2.2 Studies on the genotoxicity of gentian violet metabolites

Pentamethylpararosaniline

OEHHA identified studies on the genotoxicity of pentamethylpararosaniline, a gentian violet metabolite, as well as a small constituent of commercial gentian violet preparations. As shown in Table 7, pentamethylpararosaniline induces mutations in four strains of *Salmonella typhimurium* (TA97, TA100, TA104, and TA1535), *B. subtilis*, *E. coli*, and bacteriophage, and binds to calf thymus DNA in a cell-free system.

Table 7. Genotoxicity studies on pentamethylpararosaniline

Assay endpoint	Test system	Test article[1]	Concentration	Results	Reference
Mutagenicity	Bacteriophage T4D	Methyl violet	1.8-400 µg/ml	+	Kvelland (1983)
Mutagenicity	*Salmonella typhimurium* TA 97, TA98, TA100, and TA104	Pentamethyl-pararosaniline	0.1-5 µg/plate	+[2] TA97 and TA104, – TA98 and TA100	Aidoo et al. (1990)
Mutagenicity	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, and TA1538	Methyl violet 2B, C.I. 42535	0.32-10 µg/plate	+ TA1535 (without S9), – all other strains	Bonin et al. (1981)
Mutagenicity	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, and TA1538	Methyl violet 2B	Not reported	– (no data given)	Chung et al. (1981)
Mutagenicity	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, and TA1538	Methyl violet[3]	1-4 µg/plate	± TA1535, – all other strains	Shahin and Von Borstel (1978)
Mutagenicity	*Salmonella typhimurium* TA98 and TA100 with S9	Methyl violet	50 µg/plate	– TA98, + TA100	Yamaguchi (1988)
Mutagenicity	*B. subtilis*	Methyl violet	Not reported	+	Matsui (1980)
Mutagenicity	*E. coli Wp2s (trp, uvrA)*	Pentamethyl-pararosaniline	5 µM	+	Hass et al. (1986)
Mutagenicity	*Saccharomyces cerevisiae* strain XV185-14C	Methyl violet[3]	2-8 µg/plate	–	Shahin and Von Borstel (1978)
Mutagenicity	*Saccharomyces cerevisiae* strain 15B-II4	Methyl violet (C.I. 42535)	0-15 µg/ml	–	Zimina and Pavlenko (1990)
DNA binding	Calf thymus DNA (ctDNA)	Methyl violet	0-60 µM	+	Chi et al. (2009)

±, equivocal.
[1] As named by the study authors; confirmed to be pentamethylpararosaniline by OEHHA.
[2] Weakly positive. The results were stronger in the presence of S9.
[3] The authors referred to the chemical as "methyl violet (crystal violet; gentian violet)" in Figure 1 of the publication; however, the chemical structure in Figure 1 is of pentamethylpararosaniline chloride.

Leucogentian violet and leuco-pentamethylpararosaniline

Leucogentian violet and leuco-pentamethylpararosaniline both induced mutations in *Salmonella* TA98 in the presence of S9 (Hass et al. 1986).

Tetramethylpararosaniline isomers

The two tetramethylpararosaniline isomers, namely N,N,N',N'- and N,N,N',N''-tetramethylpararosaniline, both induced mutations in *E. coli* (Hass et al. 1986). In another study, N,N,N',N'-tetramethylpararosaniline was weakly mutagenic in *Salmonella* TA97 and TA104, and N,N,N',N''-tetramethylpararosaniline was weakly mutagenic in *Salmonella* strains TA97, TA98, TA100, and TA104 (Aidoo et al. 1990).

C.I. Basic Red 9, Michler's ketone, and formaldehyde

C.I. Basic Red 9 and Michler's ketone are microbial metabolites of gentian violet that may be produced by intestinal microflora. Both are IARC Group 2B carcinogens with positive genotoxicity data (see Section 3.3.4). Briefly, C.I. Basic Red 9 is mutagenic in bacteria and mouse lymphoma cells, tests positive for DNA damage in *E. coli* and unscheduled DNA synthesis (UDS) in mammalian cells *in vitro*, results in mutagenic urine in mice and rats, and is positive for chromosomal aberrations (CAs) in Syrian hamster embryo cells. Michler's ketone induces mutations in some strains of *Salmonella* and in mouse lymphoma L5178Y cells, DNA damage, UDS, and DNA binding in rats, CAs in Chinese hamster ovary (CHO) and CHE-3N cells, SCE in CHO cells and in bone marrow cells of mice *in vivo*, and chromosome aneuploidy in CHE-3N cells.

Formaldehyde is formed during the reductive metabolism of gentian violet (see Section 3.3.1). Formaldehyde is an IARC Group 1 carcinogen with positive genotoxicity data (IARC 2006, 2012). Positive findings of genotoxcity (e.g., mutations, DNA damage, DNA strand breaks, CAs, MN, SCE, and DNA-protein crosslinks) come from studies of exposed humans and animals, and from *in vitro* systems.

3.3.3 Animal tumor pathology

This section describes the relevant pathology details for the tumor types observed in the animal cancer bioassays of gentian violet.

Rats

Liver hepatocellular tumors

Hepatocellular adenomas were increased in male F344 rats treated with gentian violet (NCTR 1988; Littlefield et al. 1989). One hepatocellular carcinoma was observed in the 100 ppm group. Liver hepatocellular carcinomas are rare (0.7%) in untreated male F344 rats in NTP feeding studies (Haseman et al. 1985; Haseman et al. 1998) and in untreated male F344 rats tested by the NCI's Carcinogenicity Testing Program (Goodman et al. 1979).

Hepatic adenomas and carcinomas arise from the same cell type, and adenomas can progress to carcinomas (Harada et al. 1999).

Thyroid follicular cell tumors

Thyroid follicular cell adenomas and adenocarcinomas were increased in male and female F344 rats treated with gentian violet for 24 months. Thyroid follicular cell adenomas are rare in untreated male and female F344 rats, occurring at 0.39-0.9% in males and 0.17-0.5% in females in NTP and NCI two-year feeding studies (Goodman et al. 1979; Haseman et al. 1985). Thyroid follicular cell (adeno)carcinomas are rare in untreated female F344 rats, occurring at 0.28-0.4% in these studies (Goodman et al. 1979; Haseman et al. 1985). Specifically, Goodman et al. (1979) reported that thyroid follicular cell adenomas occur at 0.17% and carcinomas occur at 0.39% in untreated female F344 rats. In male F344 rats, thyroid follicular cell carcinomas were reported to be rare by Haseman et al. (1985) and Goodman et al. (1979). Thyroid gland follicular cell adenomas and (adeno)carcinomas arise from the same cell type (Botts et al. 1991).

Mononuclear cell leukemia

MNCL is a common neoplasm in aging F344 rats, and an increase of MNCL was observed at the 18-month interim sacrifice in high-dose female rats, with a significant dose-related trend across all treatment groups. Although no treatment-related increase was apparent in the animals allowed to live up to 2 years, NCTR (1988) noted that "dosing with gentian violet was significantly associated with an earlier onset and increased mortality due to leukemia".

MNCL is morphologically characterized by cells that resemble large granular lymphocytes, and can spread to multiple organs, including liver, lungs, and spleen, with splenic infiltration being the most consistent hallmark (Stromberg and Vogtsberger 1983). The spontaneous incidence of MNCL in female F344 rats in NTP two-year

studies was 20.2% during 1977-1987 and 26.8% during 1980-1989 (Caldwell 1999). The incidence of MNCL in the control female rats was 0 at 18 months and 45% at 24 months (NCTR 1988). US EPA (2012) noted that several authors have concluded that rat MNCL is similar to human natural killer (NK) cell large granular lymphocytic leukemia (Ishmael and Dugard 2006; Stromberg 1985; Thomas et al. 2007).

Mesothelioma of the testis and epididymis

Mesotheliomas of the tunica vaginalis of the testis and epididymis were observed in the treated male F344 rats at 18 months and 24 months. This site is not part of the routine necropsy, and only the tumors that were observed grossly were later confirmed microscopically.

Mesothelioma of the tunica vaginalis of the testes is a malignant neoplasm. The tumor growth can extend to the epididymides and spermatic cord with seeding of the peritoneal cavity and viscera (McConnell et al. 1992).

Clitoral gland tumors

Clitoral gland adenomas or adenocarcinomas were observed in female F344 rats at 24 months. The clitoral glands are a pair of sebaceous glands located in the inguinal region adjacent to the vagina. Their excretory ducts empty on the clitoris near the urethral opening (Yoshizawa 2018). In female rats, the growth and activity of the glands are regulated by hormones (Rudmann et al. 2012). Although there are no equivalent glands in humans, rodent clitoral glands are standard tissues that are evaluated for human risk assessment of chemicals (Rudmann et al. 2012). Both adenoma and adenocarcinoma of the clitoral gland are derived from acinar cells (Yoshizawa 2018).

Mice

Liver hepatocellular tumors

Liver hepatocellular adenomas and carcinomas were observed in gentian violet treated male and female B6C3F1 mice (Littlefield et al. 1985; NCTR 1983). Hepatocellular adenoma and carcinoma arise from the same cell type, and adenomas are considered to have the potential to progress to carcinomas, as shown by atypical cell foci of various stages of malignancy (Harada et al. 1999). NCTR (1983) mentioned that most of the hepatocellular carcinomas observed in these studies were trabecular, and a few were solid. All of the 24 liver tumors that metastasized to the lungs were of the trabecular pattern.

Harderian gland tumors

Harderian gland adenomas were increased in male and female B6C3F1 mice treated with gentian violet at 24 months (Littlefield et al. 1985; NCTR 1983).

The Harderian glands are bilateral lacrimal glands located at the posterior part of the eye of certain vertebrates (Sheldon 1994). While the Harderian glands are not present in humans, tumors of the Harderian glands in rodents are considered an indicator of cancer hazard (Huff 1992).

Reticulum cell sarcoma

Significant increases of reticulum cell sarcoma were seen in the bladder, ovary, vagina, and uterus of 600 ppm gentian violet treated female mice at 24 months, each with a dose-related trend. Although pathologically not identical, the reticular system neoplasms in mice and humans generally arise from equivalent normal cells, namely stem cells, granulocytes, reticulum cells, and plasmacytes (Dunn 1954). Mouse reticular tissue sarcoma (type A) may be sarcoma, localized monocytic leukemia, or generalized monocytic leukemia, and has been shown to be induced by chemical carcinogens (Dunn 1954). According to the pathology section of NCTR (1983), the reticulum cell sarcomas (type A) of the female genital organs were similar to the description by Dunn (1954): “The tumors were composed of sheets of elongated spindled cells with basophilic ovoid nuclei and scanty acidophilic cytoplasm, involving the wall of the vagina, cervix, and uterus.” Based on descriptions by Frith et al. (1993), reticulum cell sarcoma (type A) is an older term for neoplasms that most likely would be classified now as “histiocytic sarcoma”. However, the lack of tissue slides/blocks makes it unfeasible for NCTR pathologists to confirm a new designation (email communication with NCTR).

3.3.4 Structure activity considerations

As stated in Section 2.1, gentian violet refers to the triphenylmethane dye hexamethylpararosaniline chloride, and to commercial mixtures of triphenylmethane dyes containing > 96% hexamethylpararosaniline chloride, with < 4% pentamethylpararosaniline chloride and N,N,N',N'- and N,N,N',N''-tetramethylpararosaniline chloride, and trace levels of N,N',N''-trimethylpararosaniline chloride, each of which has a triphenylmethane core structure. OEHHA used Chemotyper (https://chemotyper.org/, Version 1.0, Revision 12976), a tool available from the US EPA for searching and highlighting chemotypes (chemical structures or subgraphs), to identify chemicals that share structural similarities with

hexamethylpararosaniline chloride. Structurally similar chemicals were chosen for structure activity comparison with gentian violet, based on the following characteristics: 1) presence of the triphenylmethane structure; 2) no methoxy groups; 3) no halogen or sulfur groups; 4) no additional aromatic rings; 5) testing for genotoxicity or animal carcinogenicity.

The six compounds are: pentamethylpararosaniline chloride, C.I. Basic Red 9 (pararosaniline hydrochloride), magenta (including magenta I, magenta II, and magenta III), malachite green chloride, leucomalachite green, and methyl green. In addition, Michler's ketone was included for structure activity comparison because it is a precursor in gentian violet production, it may be present at trace levels in commercial gentian violet (see Section 3.3.1), and it is a product of microbial metabolism of gentian violet (Chen et al. 2008; Yatome et al. 1991; Yatome et al. 1993), and thus may be produced by intestinal microflora.

Data on the genotoxicity and carcinogenicity of the selected comparison chemicals are discussed below and in Table 8. The sources of this information include the ToxNet databases (https://toxnet.nlm.nih.gov) such as Chemical Carcinogenesis Research Information System (CCRIS), Carcinogenic Potency Database (CPDB), Genetic Toxicology Data Bank (GENE-TOX), and Hazardous Substances Data Bank (HSDB), and NTP and IARC documents. No human cancer epidemiology studies were identified for any of the comparison chemicals. However, "magenta production", which involves exposure to many chemicals including C.I. Basic Red 9 as well as Magenta I, II, and III, is an IARC Group 1 carcinogen (IARC 2012). IARC (2012) found there is sufficient evidence in humans that magenta production causes urinary bladder cancer.

Pentamethylpararosaniline chloride may be a minor constituent of commercial gentian violet preparations. It is a demethylated metabolite of hexamethylpararosaniline chloride (see Section 3.3.1). No long-term carcinogenicity studies in animals were identified for this chemical. It is mutagenic in *Salmonella*, *B. subtilis*, *E. coli*, and phage T4D, and binds to calf thymus DNA (see Section 3.3.2).

C.I. Basic Red 9 (pararosaniline hydrochloride) is the fully demethylated metabolite of hexamethylpararosaniline chloride, which is also a microbial metabolite of gentian violet that may be produced by intestinal microflora. C.I. Basic Red 9 is an IARC group 2B carcinogen. It induces tumors in male and female rats and male and female mice in a variety of sites (NTP 1986). It is mutagenic in *Salmonella*, *E. coli*, and mouse lymphoma LY5178 cells, positive for DNA damage in *E. coli* and UDS in mammalian cells *in vitro*, results in mutagenic urine in mice and rats, and is positive for chromosomal aberrations (CAs) in Syrian hamster embryo cells (IARC 2010; Leifer et al. 1981).

Magenta, which has historically been used to refer to the dye mixture “basic fuchsin”, is comprised of four major constituents, namely Magenta 0 (C.I. Basic Red 9), Magenta I (rosaniline), Magenta II, and Magenta III (new fuchsin). The structures of Magenta I, II, and III are shown in Table 8. IARC (2010, 2012) considered the carcinogenicity of magenta and magenta production, and classified magenta as “possibly carcinogenic to humans” (Group 2B), based primarily on mechanistic and other relevant data. Regarding the carcinogenicity of magenta in animals, IARC (2012) noted the following:

> “Magenta was tested for carcinogenicity by oral administration in one experiment in mice (Bonser et al., 1956), one experiment in rats (Ketkar & Mohr, 1982) and one experiment in hamsters (Green et al., 1979). These studies were found to be inadequate to evaluate the carcinogenicity of magenta in experimental animals.”

Besides issues of study quality, it is unclear which one of the magenta chemicals (e.g., Magenta I, II, III, or some combination) was used in each of these magenta cancer bioassays.

Genotoxicity studies on magenta as a whole (exact composition of the products tested and the degree of purity unknown) indicate that magenta is mutagenic in *Salmonella* (IARC 2010). In addition, Magenta I has been shown to be mutagenic in *Salmonella* and *E. coli*, and positive in the rec-assay in *B. subtilis* (Fujita et al. 1976; IARC 2010; Mortelmans et al. 1986).

Malachite green chloride was tested in long-term carcinogenicity studies by NTP (2005) in female rats and mice. In female rats, NTP concluded there was “equivocal evidence of carcinogenic activity” based on the occurrence of thyroid gland follicular cell adenoma or carcinoma (combined) (0/46, 0/48, 3/47, 2/46 in control, low-, mid-, and high-dose groups, respectively) and increased incidences of hepatocellular adenoma (1/48, 1/48, 3/48, 4/48) and mammary gland carcinoma (2/48, 2/48, 1/48, 5/48) (NTP, 2005). NTP concluded there was “no evidence” of carcinogenic activity observed in the study conducted in female mice. Malachite green chloride induced DNA damage in CHO cells (Fessard et al. 1999) and in the liver of male Swiss albino mice (Donya et al. 2012), induced MN in bone marrow polychromatic erythrocytes of male rats (NTP 2004), CAs in Chinese hamster lung cells (Ishidate 1981, as cited by ECHA 2010), and CAs and sister chromatid exchange (SCE) in the bone marrow of treated Swiss albino male mice (Donya et al. 2012).

Leucomalachite green is a reduction product of malachite green chloride. It was tested in long-term carcinogenicity studies by NTP in male and female F344/N rats and female $B6C3F_1$ mice (NTP 2005). In male rats, NTP concluded there was “equivocal evidence” of carcinogenic activity based on increased incidences of interstitial cell adenoma of the

testes (37/48, 42/47, 43/48, 45/47 in control, low-, mid-, and high-dose groups, respectively) and the occurrence of thyroid gland follicular cell adenoma or carcinoma (combined) (0/47, 2/47, 1/48, 3/46). In female rats, NTP concluded there was "equivocal evidence" of carcinogenic activity based on increased incidences of hepatocellular adenoma (1/48, 3/48, 0/48, 3/48) and the occurrence of thyroid gland follicular cell adenoma or carcinoma (combined) (0/46, 1/46, 2/47, 1/48). In female mice, NTP concluded there was "some evidence" of carcinogenic activity based on increased incidences of hepatocellular adenoma or carcinoma (combined) (3/47, 6/48, 6/47, 11/47).

In addition to the NTP studies, leucomalachite green has also been tested by the NCTR in two-year feeding studies in male and female F344/N NCTR BR rats (Culp et al. 2002). It caused a marginally increased incidence of lung alveolar/bronchiolar adenomas in male rats ($p = 0.052$), with a significant positive trend (1/48, 2/47, 5/48, 6/47) (Culp et al. 2002). Leucomalachite green induced mutations in livers of treated female Big Blue B6C3F1 mice (Mittelstaedt et al. 2004) and induced DNA adducts in livers of treated male and female rats and female mice (Culp et al. 2002).

No long-term carcinogenicity studies in animals were identified for methyl green. Methyl green did not induce sex-linked recessive lethal gene mutations in *Drosophila melanogaster* (Clark 1953). It did not induce DNA damage in a rec-assay spot test in *B. subtilis* (H17A vs M45T) (Kada et al. 1972). Methyl green did not induce mutations in *Salmonella* (Chung et al. 1981).

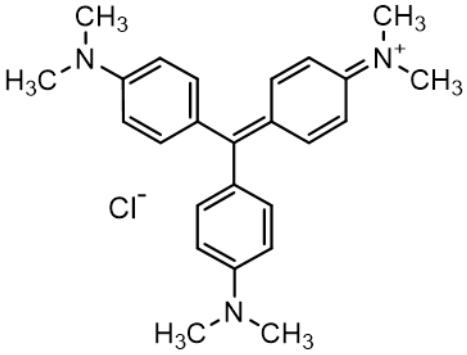
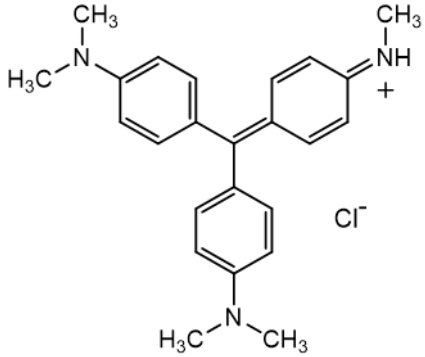
Michler's ketone (4,4'-Bis(dimethylamino)benzophenone) is a precursor in the synthesis of dyes including gentian violet (Gessner and Mayer 2000), and is also a microbial metabolite of gentian violet that may be produced by intestinal microflora (Yatome et al. 1991; Yatome et al. 1993). Michler's ketone is an IARC Group 2B carcinogen (IARC 2010). In the cancer bioassays conducted by NCI (1979), dietary administration of Michler's ketone induced tumors in male and female rats, and male and female mice (NCI 1979).

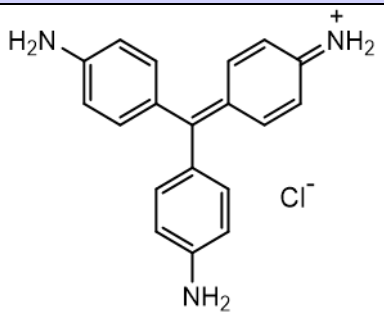
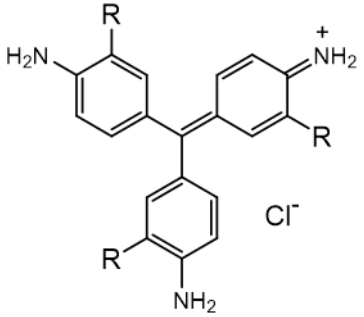
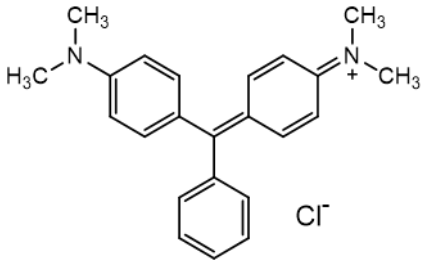
Michler's ketone is mutagenic in some strains of *Salmonella* (Dunkel and Simmon 1980; Dunkel et al. 1985; Kamber et al. 2009; NTP 2018; Tennant et al. 1986) and in mouse lymphoma L5178Y cells (Mitchell et al. 1988; Myhr and Caspary 1988). It tested positive in several *in vivo* DNA damage assays, induced UDS in male F344 rats (Mirsalis et al. 1989), and induced liver DNA damage in rats (Kitchin and Brown 1994; Parodi et al. 1982). Michler's ketone bound to liver DNA in rats (Scribner et al. 1980) and liver and kidney DNA in phenobarbital-pretreated rats (Struck et al. 1981). It induced CAs in CHO cells and CHE-3N cells (Lafi et al. 1986; NTP), SCE in CHO cells

(NTP 2018) and in bone marrow of Swiss mice *in vivo* (Parodi et al. 1982), and increased the level of chromosome aneuploidy in CHE-3N cells (Lafi et al. 1986).

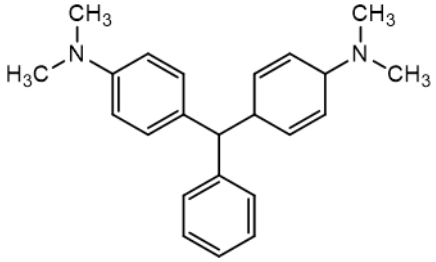
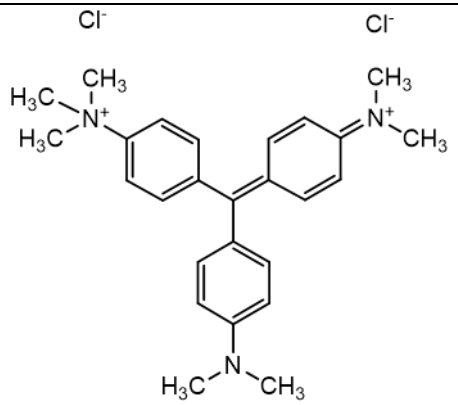
As summarized in Table 8, seven chemicals that are structurally related to hexamethylpararosaniline chloride were considered for comparison. All of the comparison chemicals were tested for mutagenicity and all except methyl green tested positive. Three of the comparison chemicals, namely C.I. Basic Red 9, malachite green chloride, and Michler's ketone were tested for chromosomal effects and all three tested positive. All comparison chemicals were tested for DNA damage/binding, and all but methyl green tested positive.

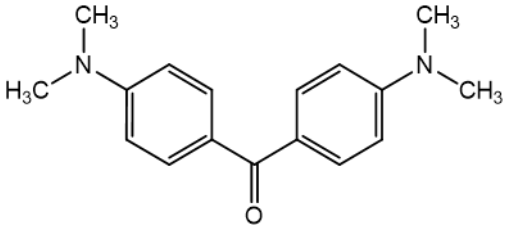
Table 8. Structure activity comparison between gentian violet and seven structurally related chemicals

Chemical	Structure	Genotoxicity			Tumors in animal bioassays
		Mutagenicity	Chromosomal effects	DNA damage/ DNA binding	
Gentian violet (Hexamethylpararosaniline chloride) CAS# 548-62-9		+ *Salmonella* + frameshift mutations in *E. coli* – *Saccharomyces cerevisiae* – CHO-K1-BH_4 cells ± CHO-AS52 cells – Sex-linked recessive lethal gene mutation in *Drosophila melanogaster*	+ CAs in CHO, human HeLa cells and cultured lymphocytes, and other mammalian cell lines + chromosomal breakage in CHO and human peripheral blood cells; – SCE in chicken embryo cells *in vivo* – chromosomal damage in mice *in vivo*	+ DNA damage in *B. subtilis, E. coli,* and mouse lymphocytes + DNA binding to calf thymus DNA, bacteriophage DNA, and bacterial DNA + binding to mitotic figures in human oral mucosal tissue *in vitro* (+) gene amplification in a SV40-transformed hamster cell line	MR: liver, thyroid, mesothelioma of the testis and epididymis FR: thyroid, MNCL, clitoral gland MM: liver, Harderian gland FM: liver, Harderian gland, reticulum cell sarcomas (type A) (likely histiocytic sarcoma) in bladder, ovary, uterus, and vagina
Pentamethylpararosaniline chloride (Methyl Violet) CAS# 8004-87-3 May comprise < 4 % of commercial gentian violet preparations Metabolite of hexamethyl-pararosaniline chloride		+ bacteriophage T4D + *Salmonella* + *B. subtilis* + *E. coli* – *S. cerevisiae*	NT	+ binding with calf thymus DNA in a cell-free system	NT

Chemical	Structure	Genotoxicity			Tumors in animal bioassays
		Mutagenicity	Chromosomal effects	DNA damage/ DNA binding	
C.I. Basic Red 9 (pararosaniline hydrochloride) CAS# 569-61-9 **(P65 carcinogen, IARC 2B)** Microbial metabolite of hexamethylpararosaniline chloride, expected metabolite in mammals		+ *E. coli* + *Salmonella* + *Tk* locus in mouse lymphoma cells + mutagenicity of urine from mice and rats fed C.I. Basic Red 9	+ CAs in Syrian hamster embryo cells – SCE in CHO cells	+ DNA damage in *E. coli* + UDS in Syrian hamster hepatocytes and rat primary hepatocytes	MR: liver, skin, subcutaneous tissue, thyroid, zymbal gland FR: subcutaneous tissue, thyroid, zymbal gland, (±) mammary gland MM: liver FM: adrenal gland, liver, (±) lymphoma
Magenta **(includes Magenta I, II, & III)**[5] **(IARC 2B)**		+ *Salmonella* + *E. coli* WP2 (trp-)	NT	+ *B. subtilis* strains H17A vs M45T	No adequate studies (IARC 2012)
Malachite Green Chloride CAS# 569-64-2		– *Salmonella* – *hgprt* locus mutation frequency in FM livers *in vivo* – *cII* mutant frequency in FM livers *in vivo*	+ CAs in Chinese hamster lung cells + CAs and SCE in mouse bone marrow *in vivo*	+ MN in MR – MN in MM, FM + DNA damage in CHO cells + DNA fragmentation in mouse	(±) FR: mammary gland, liver, thyroid

[5] The chemical structure refers to "Magenta I" when one of the R groups equals $–CH_3$, "Magenta II" when two R groups equal $–CH_3$, and "Magenta III" when all three R groups equal $–CH_3$.

Chemical	Structure	Genotoxicity			Tumors in animal bioassays
		Mutagenicity	Chromosomal effects	DNA damage/ DNA binding	
				hepatocytes *in vivo* + DNA adduct formation in rats and mice *in vivo*	
Leucomalachite Green CAS# 129-73-7		– *Salmonella* – *hgprt* locus mutation frequency in FR, FM livers *in vivo* + *cII* mutant frequency in FM livers *in vivo* (– in FR)	NT	+ MN in peripheral blood of FM – MN in FR + DNA adduct formation in rats and mice *in vivo*	MR: lung, (±) testis interstitial adenoma, (±) thyroid FR: (±) liver, (±) thyroid FM: liver
Methyl Green CAS# 82-94-0; 54327-10-5		– Sex-linked recessive lethal gene mutation in *Drosophila melanogaster*	NT	– DNA damage in *B. subtilis*	NT

Chemical	Structure	Genotoxicity			Tumors in animal bioassays
		Mutagenicity	Chromosomal effects	DNA damage/ DNA binding	
Michler's Ketone CAS# 90-94-8 (**Prop 65 carcinogen, IARC 2B)** Microbial metabolite of hexamethylpararosaniline chloride		+ *Salmonella* – *E. coli* WP2 urvA + mouse lymphoma L5178Y cells	+ CAs in CHO and CHE-3N cells + SCE in CHO cells + aneuploidy in CHE-3N cells + SCE in mouse bone marrow *in vivo*	– *B. subtilis* – HL-60 cells + UDS in MR *in vivo* + liver DNA damage in rats *in vivo* + binding to liver and kidney DNA in MR *in vivo*	MR: liver FR: liver MM: Hemangiosarcoma; fibrosarcomas or sarcomas of the skin and subcutaneous tissue FM: liver

MR, male rats; FR, female rats; MM, male mice; FM, female mice; H, hamsters; +, positive; (+), weakly positive; ±, equivocal; –, negative; NT, not tested. CAs, chromosomal aberrations; CHO, Chinese hamster ovary; MN, micronuclei; MNCL, mononuclear cell leukemia; SCE, sister chromatid exchange; UDS, unscheduled DNA synthesis.

The tumor site information for the comparison chemicals is summarized in Table 9. Three of the tumor types observed to be increased in animal studies of gentian violet, namely liver hepatocellular tumors, thyroid follicular cell tumors, and Harderian gland tumors, were also increased in animal studies of some of the comparison chemicals. Specifically, increases in hepatocellular tumors were seen with C.I. Basic Red 9, leucomalachite green, and Michler's ketone (with equivocal findings for malachite green chloride); thyroid follicular cell tumors were seen with C.I. Basic Red 9 (with equivocal findings for malachite green chloride and leucomalachite green); and Harderian gland tumors were seen with C.I. Basic Red 9.

Table 9. Animal tumor sites for gentian violet and several structurally similar chemicals[1]

Chemical \ Tumor Site	Liver (hepato-cellular)	Thyroid (follicular cell)	Harderian gland	Testes	Lung	Mammary gland	Other
Gentian Violet	MR MM FM	MR FR	MM FM	MR[2]			Clitoral gland (FR) MNCL[3] (FR) Reticulum cell sarcoma (type A) in bladder, ovary, uterus, and vagina (likely histiocytic sarcoma) (FM)
C.I. Basic Red 9 monohydrochloride	MR MM FM	MR FR	FM		FM	(±) FR	Skin (MR) Subcutaneous fibroma (MR, FR) Zymbal gland (MR, FR) Adrenal gland pheochromocytoma (FM) (±) malignant lymphoma (FM)
Malachite Green Chloride	(±) FR	(±) FR				(±) FR	
Leucomalachite Green	(±) FR FM	(±) MR (±) FR		(±)MR[4]	MR		
Michler's ketone	MR FR FM						Hemangiosarcoma (MM)

MR, male rats; FR, female rats; MM, male mice; FM, female mice; (±), equivocal.

[1] Pentamethylpararosaniline chloride and methyl green have not been tested in long-term carcinogenicity studies in animals, and magenta has not been adequately tested for carcinogenicity in animals.

[2] Mesothelioma of the testis and epididymis

[3] Significant increase at 600 ppm with significant dose-related trend at 18 months, but not at two years.

[4] Interstitial cell adenoma of the testes

3.3.5 ToxCast high-throughput screening assays

ToxCast™ is a chemical prioritization research program developed by the US EPA (Dix et al. 2007; Judson et al. 2010; Kavlock et al. 2012). It is a multi-year project that launched in 2007. ToxCast utilizes various *in vitro* and zebrafish systems to identify chemical activity in a battery of high-throughput screening (HTS) assays. As of 2018, more than 9,000 chemicals have been tested and there are more than 1,000 high-throughput assays in the ToxCast database.

This section highlights the ToxCast HTS assays in which gentian violet and pentamethylpararosaniline chloride (a metabolite of gentian violet which can be present at <4% in commercial gentian violet preparations) were active. OEHHA has searched the ToxCast database via the Interactive Chemical Safety for Sustainability (iCSS) Dashboard (https://actor.epa.gov/dashboard/, v2, accessed on 4/24/2018), and obtained chemical activity data for gentian violet and pentamethylpararosaniline chloride[6]. OEHHA found that gentian violet was active in 273 of the 794 ToxCast assays in which it was tested, and pentamethylpararosaniline chloride was active in 21 of the 28 assays in which it was tested (Table 10). The purity of the gentian violet used in these assays was typically over 90% hexamethylpararosaniline chloride. The purity of pentamethylpararosaniline chloride used in the ToxCast assays was reported to be < 14%.

Table 10. Overview of ToxCast HTS assay activity for gentian violet and pentamethylpararosaniline chloride

Chemical	Gentian violet	Pentamethylpararosaniline chloride
Number of active assays / tested assays [1]	273/794	21/28
Range of AC_{50} values (µM) in active assays	0.000841-80.1	0.294-32.3

[1] Does not include results classified by US EPA as 'background measurement' assays (i.e., artifact fluorescence, baseline controls, and internal markers).
AC_{50}: the concentration that induces a half-maximal assay response.

[6] Identified in ToxCast by CAS number 8004-87-3.

Aligning active gentian violet ToxCast Assays to IARC's key characteristics of carcinogens

In 2014, the IARC working group for Monograph Volume 112 mapped the ToxCast assay end-points available at that time to the key characteristics of carcinogens (IARC 2017; Smith et al. 2016). IARC has since updated their assay mapping and OEHHA obtained IARC's latest table (Houck, K., email communication, May 24, 2018), which maps a total of 299 assay end-points to seven of the ten key characteristics. OEHHA used IARC's latest table to map the active ToxCast assays for gentian violet to the key characteristics of carcinogens. Seventy-two assays in which gentian violet was active mapped to five of the ten key characteristics of carcinogens (Figure 3 and Appendix C).

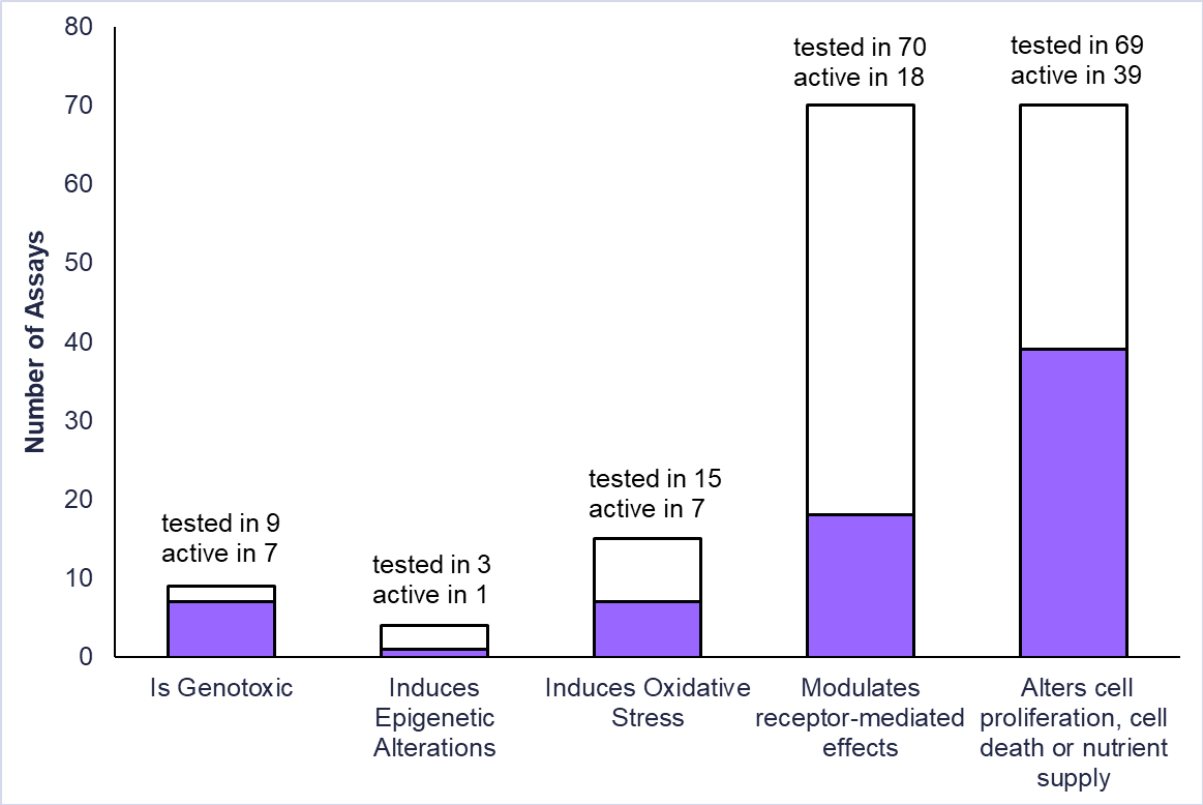


Figure 3. Numbers of ToxCast assays in which gentian violet was active and tested, corresponding to selected key characteristics of carcinogens

Bars indicate the number of assays mapped to specific key characteristics for which gentian violet was tested. Filled bars: number of assays mapped to specific key characteristics in which gentian violet was active.

Figure 3 shows the distribution of ToxCast assays in which gentian violet was active and tested, mapped to each characteristic. Specifically, gentian violet:

- *Is genotoxic* – Gentian violet was active in seven assays targeting cellular TP53 induction. Although not a direct measurement of genotoxicity, TP53 is a central player in cellular responses to DNA damage and can be activated by genotoxic stress (Speidel 2015).
- *Induces epigenetic alterations* – Gentian violet was active in one assay targeting the activation of Sp1 transcription factor, which is overexpressed in many cancers as a key regulator in cancer progression. Sp1 is associated with many cancer-related pathways including epigenetic silencing (Beishline and Azizkhan-Clifford 2015).
- *Induces oxidative stress* – Gentian violet was active in three assays measuring oxidative stress and four assays targeting the activation of oxidative stress-related transcription factors: metal-regulatory transcription factor 1 (MTF1), nuclear factor erythroid 2-like 2 (NFE2L2 or Nrf2) (two assays), and heat shock transcription factor 1 (HSF1).
- *Modulates receptor-mediated effects* – Gentian violet was active in 18 assays targeting receptor-mediated effects. The receptors targeted by gentian violet include:
    - Androgen receptor (AR) (four assays);
    - Estrogen receptor alpha (ERα) (four assays);
    - Hepatocyte nuclear factor 4; alpha (HNF4A);
    - Nuclear receptor subfamily 1, group H, member 4, (NR1H4);
    - Nuclear receptor subfamily 1, group I, member 2 (NR1I2);
    - Nuclear receptor subfamily 3, group C, member 1 (NR3C1, a glucocorticoid receptor) (two assays);
    - Peroxisome proliferator-activated receptor gamma (PPARγ) (three assays);
    - Thyroid hormone receptor, beta (THRB); and
    - Vitamin D receptor (VDR) (activity tested via the reporter gene CYP24A1, which carries VDRE in its promoter).
- *Alters cell proliferation, cell death or nutrient supply* – Gentian violet was active in 38 assays measuring cytotoxicity by a wide variety of assay formats in cell lines or primary human cells. Specifically, gentian violet was active in assays mapped to cell cycle (mitotic arrest), cell conformation changes, as well as mitochondrial toxicity by loss of mitochondria membrane potential. Notably, one of the assays detects increased transcription activity at AP-1 sites, which reflects activities of proto-oncogenes such as c-Jun and c-Fos (van Dam and Castellazzi 2001).

Iyer et al. (2018) analyzed the chemicals tested in ToxCast Phases I and II for activity in a subset of cancer pathway-related assays. In this analysis, gentian violet was in the

top 5% of active chemicals, and was ranked seventh out of the 1,061 chemicals examined.

Intended target families for gentian violet and pentamethylpararosaniline chloride

The ToxCast HTS assays cover a broad range of potential toxicity mechanisms that are not limited to carcinogenicity, and gentian violet tested positive in many of these other assays. Biological process categories, or "intended target families" for all active assays reported for gentian violet and pentamethylpararosaniline chloride, were obtained from the iCSS ToxCast Dashboard (accessed on 4/24/2018) and are presented in Tables 11 and 12, respectively. Additional detailed information about these assays, such as specific molecular targets and AC_{50} values, are available in Appendix C.

Table 11. Intended target families of the active ToxCast HTS assays for gentian violet

Intended Target Family[1]	Number of active assays
GPCR	55
Cytokine	41
Cell cycle, cytotoxicity	40
Nuclear receptor	32
DNA binding	25
Cell adhesion molecules	16
Cytochrome P450	9
Ion Channel	9
Protease	9
Transporter	9
Cell morphology	8
Oxidoreductase	5
Developmental defect (in zebrafish)	3
Esterase	3
Kinase	3
Growth factor	2
Miscellaneous protein	2
Protease inhibitor	2
Total	273

[1] Intended assay target families were assigned to each assay by the US EPA.

As shown in Table 11, gentian violet was active in 273 ToxCast assays, including a subset of 72 assays that were mapped to key characteristics of carcinogens and described earlier. These 273 assays are related to 17 biological processes or intended target families, as shown in the table. The largest proportions of assays in which gentian violet was active were the GPCR, cytokine, and cell cycle/cytotoxicity target families.

Table 12. Intended target families of the active ToxCast HTS assays for pentamethylpararosaniline chloride

Intended Target Family[1]	Number of active assays
Cell cycle, cytotoxicity	6
Cytochrome P450	1
DNA binding	6
Nuclear receptor	8
Total	21

[1] Intended assay target families were assigned to each assay by the US EPA.

As shown in Table 12, pentamethylpararosaniline chloride was active in 21 assays related to four different biological processes or intended target families, namely cytotoxicity, cytochrome P450s, DNA binding, and nuclear receptors.

4. MECHANISMS

Gentian violet may act via multiple mechanisms, which can be grouped according to the key characteristics of carcinogens described by Smith et al. (2016). These mechanisms include being electrophilic or forming electrophilic metabolites, genotoxicity, oxidative stress induction, and receptor-mediated effects (Table 13).

Table 13. Ten key characteristics of carcinogens (taken from Smith et al. 2016)

Characteristic	Example of relevant evidence
1. Is electrophilic or can be metabolically activated	Parent compound or metabolite with an electrophilic structure (*e.g.,* epoxide, quinone), formation of DNA and protein adducts
2. Is genotoxic	DNA damage (DNA strand breaks, DNA–protein cross-links, UDS), intercalation, gene mutations, cytogenetic changes (*e.g.,* CAs, MN)
3. Alters DNA repair or causes genomic instability	Alterations of DNA replication or repair (*e.g.,* topoisomerase II, base-excision or double-strand break repair)
4. Induces epigenetic alterations	DNA methylation, histone modification, microRNA expression
5. Induces oxidative stress	Oxygen radicals, oxidative stress, oxidative damage to macromolecules (*e.g.,* DNA, lipids)
6. Induces chronic inflammation	Elevated white blood cells, myeloperoxidase activity, altered cytokine and/or chemokine production
7. Is immunosuppressive	Decreased immunosurveillance, immune system dysfunction
8. Modulates receptor-mediated effects	Receptor inactivation/activation (*e.g.,* ER, PPAR, AhR) or modulation of endogenous ligands (including hormones)
9. Causes immortalization	Inhibition of senescence, cell transformation
10. Alters cell proliferation, cell death, or nutrient supply	Increased proliferation, decreased apoptosis, changes in growth factors, energetics and signaling pathways related to cellular replication or cell cycle control, angiogenesis

AhR, aryl hydrocarbon receptor; ER, estrogen receptor; PPAR, peroxisome proliferator–activated receptor. Any of the 10 characteristics in this table could interact with any other (e.g., oxidative stress, DNA damage, and chronic inflammation), which when combined provides stronger evidence for a cancer mechanism than would oxidative stress alone.

Gentian violet is a direct-acting electrophile that reacts with DNA and other nucleophiles (Muller and Gautier 1975; NCTR 1983). Gentian violet also forms electrophilic metabolites. Free nitrogen- or carbon-centered radicals can be formed during metabolic N-demethylation and reduction reactions and have been observed *in vitro* in peroxidase-catalyzed reactions, in incubations with cytochrome P450 enzymes in rat microsomes or *T. cruzi* cells, and as a result of photoinduction in cell-free systems. Gentian violet is used as a stain for nuclei (see Section 2.3). Numerous studies have

shown that gentian violet can bind to DNA, specifically to adjacent AT nucleotide pairs (Muller and Gautier 1975; see also section 3.3.2 Genotoxicity). The binding occurs externally and causes severe kinking and or bending accompanied by a coupled unwinding of the helix (Wakelin et al. 1981). The ability to form adducts to nucleic acids and proteins is a common property of electrophilic and/or metabolically activated human carcinogens.

Gentian violet is genotoxic. Gentian violet has demonstrated bacterial mutagenicity in *Salmonella* and *E. coli*, clastogenicity in various test systems, and DNA binding and damage in human and rodent cells. In addition, gentian violet is metabolized to several mutagenic metabolites (pentamethylpararosaniline, N,N,N',N'- and N,N,N',N''-tetramethylpararosaniline, leucogentian violet and leuco-pentamethylpararosaniline) and to the genotoxic carcinogens formaldehyde, pararosaniline hydrochloride (C.I. Basic Red 9[7]), and Michler's ketone[8].

Multiple lines of evidence indicate that gentian violet induces oxidative stress. In cell-free systems gentian violet has been shown to generate reactive oxygen species, converting oxygen to superoxide anion and hydrogen peroxide in the presence of visible light, and in HRP-catalyzed reactions (Reszka et al. 1986; Gadelha et al. 1992). Reactive oxygen species and other free radicals arising from xenobiotic metabolism may play key roles in many of the processes necessary for the conversion of normal cells to cancer cells. In addition, several ToxCast HTS assays showed that gentian violet induces oxidative stress, and activates transcription factors such as Nrf2, whose activation is an indicator of cellular antioxidant response.

Gentian violet modulates receptor-mediated effects. Gentian violet was active in 18 ToxCast HTS assays that were mapped to the key characteristic of carcinogens "modulates receptor-mediated effects" by IARC. Examples of the receptors that are activated by gentian violet include the androgen receptor (AR), estrogen receptor alpha (ERα), peroxisome proliferator-activated receptor gamma (PPARγ), and the thyroid hormone receptor beta (THRB). Receptor-mediated activation most often results in changes in gene transcription.

[7] Expected product of mammalian metabolism. A product of microbial metabolism, it may be produced by intestinal microflora.
[8] A product of microbial metabolism, it may be produced by intestinal microflora.

5. REVIEWS BY OTHER AGENCIES

Gentian violet has not been classified as to its potential carcinogenicity by the US Environmental Protection Agency (EPA), the US Food and Drug Administration (FDA), the National Toxicology Program (NTP), the National Institute for Occupational Safety and Health (NIOSH), or the International Agency for Research on Cancer (IARC).

FDA currently permits the use of gentian violet in wound dressings (Edwards 2016; FDA 2016). However, FDA determined that the use of gentian violet in animal feed is not "generally recognized as safe" and considers animal feed containing gentian violet as adulterated (21CFR 589.1000; 21CFR 500.29; 21CFR 500.30), based on possible carcinogenicity concerns arising from structural similarities to known animal and human carcinogens and its mutagenicity. FDA noted that "Gentian violet is a suspected carcinogen, a probable mutagen, and a potent clastogen" (GAO 1980). FDA includes gentian violet in its routine testing of aquaculture drug residues and has issued import alerts (FDA 2008). FDA also mentioned "Gentian violet is not permitted as a color in food or feed in the United States. Studies at the National Center for Toxicological Research have shown gentian violet to be a carcinogen for laboratory animals." in the Compliance Policy Guide Section 578.600, "Unapproved Additives for Exported Grains" (FDA 1995).

Although not formally reviewed by the NTP, gentian violet was mentioned in the NTP (2005) technical report on the carcinogenicity of malachite green chloride and leucomalachite green, and referred to as a "carcinogenic dye" (NTP 2005, page 63). In addition, the structural similarity of malachite green to gentian violet was cited as part of the rationale for nomination by the FDA of malachite green and leucomalachite green for carcinogenesis testing by NTP.

Gentian violet was also evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA 2014) for its suitability as to whether or not an acceptable daily intake (ADI) could be established and whether the continued use of gentian violet in food-producing animals is safe for humans. The Committee concluded that "it is inappropriate to set an ADI for gentian violet because it is genotoxic and carcinogenic" (JECFA 2014).

The Australian Pesticides and Veterinary Medicines Authority (APVMA) of the National Health and Medical Research Council (NHMRC) cancelled the registrations and relevant approvals of products containing crystal (gentian) violet after a 1994 special review of crystal (gentian) violet (APVMA 2014). The review found that crystal (gentian)

violet posed a potential risk to public health. According to APVMA, “crystal violet “demonstrated carcinogenic/tumorigenic effects in mice in life-span studies. Hepatocellular carcinoma, adenoma of the Harderian gland and Type A reticular cell sarcomas in multiple sites were found in 18–24-month-old mice, and *in vitro* studies revealed that crystal (gentian) violet is a mutagen and clastogen”.

6. SUMMARY AND CONCLUSIONS

6.1 Summary of Evidence

One hospital-based study conducted in Brazil and published in Portuguese by De Sousa et al. (1989) identified 37 individuals that had received transfusions of blood treated with gentian violet in the past (i.e., 2-27 years previously), based on patient recall (i.e., transfused blood appeared purple). The researchers examined the medical records of these 37 individuals and determined that 26 out of the 37 had confirmed benign or malignant neoplastic lesions. This study has a number of limitations, including lack of information on the site and type of neoplastic lesions observed, lack of control subjects, and the likely presence of selection bias and confounding factors.

One case report published in German described a case of lymphocytic leukemia diagnosed in a 57-year old man five months after he was exposed to ink containing gentian violet, as a result of accidentally stabbing his hand with an ink pen (Schaeppi 1955). The author assumed a causal link between the injury and the leukemia; however, the report provided no information on the extent to which exposure to gentian violet occurred or on the health status of the individual (e.g., results of complete blood cell count or blood work) prior to the accident.

Long-term carcinogenicity studies of gentian violet have been conducted in rats and mice. Tumors were observed in two studies in rats and two studies in mice. These findings are as follows:

Liver tumors

- In the male F344 rats exposed to gentian violet *in utero*, during lactation, and via feed post-weaning for up to 24 months (Littlefield et al. 1989; NCTR 1988), the incidence of hepatocellular adenoma was significantly increased in the highest dose group by pairwise comparison with controls, with a significant dose-related trend. In addition, one hepatocellular carcinoma was observed in the low-dose group.

- In the male B6C3F1 mice exposed to gentian violet in feed for up to 24 months (Littlefield et al. 1985; NCTR 1983), the incidence of hepatocellular adenoma was significantly increased in the mid- and high-dose groups by pairwise comparison with controls, with a significant dose-related trend. Hepatocellular carcinoma was significantly increased in the high-dose group by pairwise comparison with controls, with a significant dose-related trend.
- In the female B6C3F1 mice exposed to gentian violet in feed for up to 24 months (Littlefield et al. 1985; NCTR 1983), the incidences of hepatocellular adenoma and carcinoma were both significantly increased in the mid- and high-dose groups by pairwise comparison with controls, with significant dose-related trends.

Thyroid tumors

- In the male F344 rats exposed to gentian violet *in utero*, during lactation, and via feed post-weaning for up to 24 months (Littlefield et al. 1989; NCTR 1988), the incidence of thyroid gland follicular cell adenocarcinoma was significantly increased in the low- and high-dose groups by pairwise comparison with controls, with a significant dose-related trend. The incidence of thyroid gland follicular cell adenoma or adenocarcinoma combined was significantly increased in the highest dose group by pairwise comparison with controls, with a significant dose-related trend.
- In the female F344 rats exposed to gentian violet *in utero*, during lactation, and via feed post-weaning for up to 24 months (Littlefield et al. 1989; NCTR 1988), the incidences of thyroid gland follicular cell adenocarcinoma and adenoma or adenocarcinoma combined were significantly increased in the mid- and high-dose groups by pairwise comparison with controls, with significant dose-related trends. These tumors are rare in untreated female F344 rats.

Testis and epididymis tumors

- In the male F344 rats exposed to gentian violet *in utero*, during lactation, and via feed post-weaning for up to 24 months (Littlefield et al. 1989; NCTR 1988), a dose-related increase in mesothelioma of the testis and epididymis was observed in the mid- and high-dose groups, and an increase was also seen at these dose groups in the 18-month interim sacrifice groups.

Mononuclear cell leukemia

- In the female F344 rats exposed to gentian violet *in utero*, during lactation, and via feed post-weaning for 18 months (Littlefield et al. 1989; NCTR 1988), the incidence of mononuclear cell leukemia was significantly increased in the highest dose group by pairwise comparison with controls, with a significant dose-related trend. Although no treatment-related increase in MNCL was apparent in animals at 24 months, NCTR (1988) stated that "dosing with gentian violet was significantly associated with an earlier onset and increased mortality due to leukemia".

Clitoral gland tumors

- In the female F344 rats exposed to gentian violet *in utero*, during lactation, and via feed post-weaning for up to 24 months (Littlefield et al. 1989; NCTR 1988), a dose-related increase in clitoral gland adenoma or adenocarcinoma combined was observed in the mid- and high-dose groups.

Harderian gland tumors

- In the male B6C3F1 mice exposed to gentian violet in feed for up to 24 months (Littlefield et al. 1985; NCTR 1983), the incidence of Harderian gland adenoma was significantly increased in the mid- and high-dose groups by pairwise comparison with controls, with a significant dose-related trend.
- In the female B6C3F1 mice exposed to gentian violet in feed for up to 24 months (Littlefield et al. 1985; NCTR 1983), the incidence of Harderian gland adenoma was significantly increased in all three treated groups by pairwise comparison with controls, with a significant dose-related trend.

Reticulum cell sarcoma (type A), likely histiocytic sarcoma

- In the female B6C3F1 mice exposed to gentian violet in feed for up to 24 months (Littlefield et al. 1985; NCTR 1983), the incidence of reticulum cell sarcoma (histiocytic sarcoma) was significantly increased in the mid- and high-dose groups by pairwise comparisons with controls, with a significant dose-related trend, in each of the following tissues:
    - Bladder
    - Ovaries
    - Uterus
    - Vagina

Metabolism of gentian violet can occur by both oxidative and reductive processes, and several of the metabolites formed are mutagenic and/or carcinogenic. Oxidative metabolites of gentian violet have been measured in *in vivo* studies of mice, rats, chickens, and microbes, and *in vitro* studies with liver microsomes isolated from mice, rats, guinea pigs, hamsters, and chickens. Reductive metabolites have been measured *in vivo* in studies of mice, rats, fish and intestinal microflora isolated from rats, mice, and humans. Free radicals can be formed by either reductive or oxidative metabolism, with a carbon-centered free radical formed during reductive metabolism and a nitrogen-centered radical formed during oxidative metabolism. Carcinogenic metabolites include the known carcinogens formaldehyde, C.I. Basic Red 9, and Michler's ketone (the latter two are products of microbial metabolism and may be produced by intestinal microflora). Other mutagenic metabolites include pentamethylpararosaniline, N,N,N',N'- and N,N,N',N''-tetramethylpararosaniline, which are products of oxidative metabolism, and leucogentian violet and leuco-pentamethylpararosaniline, which are products of reductive metabolism.

Gentian violet has tested positive for a number of genotoxicity endpoints:

- Mutations in *Salmonella typhimurium* TA97, TA98, TA100, TA104, and TA1535
- Mutations and DNA damage in *E. coli*
- DNA damage in *B. subtilis*
- DNA damage in mouse lymphocytes
- CAs in CHO, human lymphocytes and HeLa cells, and other mammalian cells
- Chromosome breakage in CHO and human peripheral blood cells
- Binding to chromosomes undergoing mitosis ("mitotic figures") in human oral mucosa tissue
- Binding to bacterial and bacteriophage DNA
- Binding to cell-free calf thymus DNA and synthetic polynucleotides
- Gene amplification in a SV40-transformed hamster cell line

There is also evidence for the genotoxicity of several metabolites of gentian violet:

- Pentamethylpararosaniline chloride (both a metabolite, and a small constituent of commercial gentian violet preparations):
    - Mutations in four strains of *Salmonella typhimurium* (TA97, TA100, TA104, and TA1535), and in *B. subtilis, E. coli,* and bacteriophage T4D
    - Binding to calf thymus DNA
- Leucogentian violet: mutations in *Salmonella typhimurium*
- Leuco-pentamethylpararosaniline: mutations in *Salmonella typhimurium*
- N,N,N',N'-tetramethylpararosaniline: mutations in *Salmonella typhimurium* and *E. coli*

- N,N,N',N''-tetramethylpararosaniline: mutations in *Salmonella typhimurium* and *E. coli*
- C.I. Basic Red 9 (a microbial metabolite of gentian violet that may be produced by intestinal microflora):
    - Mutations in *Salmonella typhimurium*, *E. coli* and mouse lymphoma cells
    - Mutagenic urine in mice and rats
    - DNA damage in *E. coli*
    - UDS in mammalian cells *in vitro*
    - CAs in Syrian hamster embryo cells
- Michler's Ketone (a microbial metabolite of gentian violet that may be produced by intestinal microflora):
    - Mutations in some strains of *Salmonella typhimurium* and in mouse lymphoma L5178Y cells
    - DNA damage, UDS, and DNA binding in rats
    - CAs in CHO and CHE-3N cells
    - SCE in CHO cells and in bone marrow cells of mice
    - Chromosome aneuploidy in CHE-3N cells
- Formaldehyde:
    - Mutations, DNA damage, DNA strand breaks, CAs, MN, SCE, and DNA-protein crosslinks in various *in vivo* and *in vitro* systems, including exposed humans

The biological activity of gentian violet was compared to seven structurally related compounds: pentamethylpararosaniline chloride, C.I. Basic Red 9 (pararosaniline hydrochloride), magenta (including magenta I, magenta II, and magenta III), malachite green chloride, leucomalachite green, methyl green, and Michler's ketone. Of these seven comparison compounds, three are classified by IARC as Group 2B carcinogens (C.I. Basic Red 9, Michler's ketone, and Magenta), and two of the three are also listed as Proposition 65 carcinogens (C.I. Basic Red 9 and Michler's ketone). Three of the tumor types observed to be increased in animal studies of gentian violet were also increased in animal studies of some of the comparison chemicals. Specifically, increases in hepatocellular tumors were seen with C.I. Basic Red 9, leucomalachite green, and Michler's ketone (with equivocal findings for malachite green chloride); thyroid follicular cell tumors were seen with C.I. Basic Red 9 (with equivocal findings for malachite green chloride and leucomalachite green); and Harderian gland tumors were seen with C.I. Basic Red 9. All of the comparison chemicals were tested for genotoxicity, and all except methyl green tested positive. Specifically, all except methyl green tested positive for mutagenicity and DNA damage or binding, and all three of the comparison chemicals that were tested for chromosomal effects tested positive (C.I. Basic Red 9, malachite green chloride, and Michler's ketone).

Gentian violet was tested in 794 high-throughput screening assays in the US EPA ToxCast database, and was active in 273 assays. These 273 assays have been categorized by US EPA as being related to 17 biological processes or intended target families. The largest proportions of assays in which gentian violet was active were related to the GPCR, cytokine, and cell cycle/cytotoxicity target families. In a separate analysis, OEHHA applied information from IARC that maps ToxCast assay endpoints to the key characteristics of carcinogens, and found that 72 of the 273 assays in which gentian violet was active mapped to five of the key characteristics of carcinogens. Specifically, seven of the nine assays mapped to “is genotoxic” were active, one of the three assays mapped to “induces epigenetic alterations” was active, seven of the 15 assays mapped to “induces oxidative stress” were active, 18 of the 70 assays mapped to “modulates receptor-mediated effects” were active, and 39 of the 69 assays mapped to “alters cell proliferation, cell death, or nutrient supply” were active.

Gentian violet may act via multiple mechanisms, including being electrophilic or forming electrophilic metabolites, genotoxicity, oxidative stress induction, and receptor-mediated effects.

- Gentian violet is a direct acting electrophile that reacts with DNA and other nucleophiles. Gentian violet also forms electrophilic metabolites, such as free nitrogen- or carbon-centered radicals formed during metabolic N-demethylation and reduction reactions.
- Gentian violet is genotoxic, as summarized above.
- Gentian violet induces oxidative stress by forming reactive oxygen species. In addition, gentian violet is active in seven ToxCast HTS assays that have been mapped to this key characteristic.
- Gentian violet can modulate receptor-mediated effects, based on its activity in 18 ToxCast HTS assays that have been mapped to this key characteristic.

6.2 Conclusions

The evidence for the carcinogenicity of gentian violet comes from:

- Studies in male and female rats and mice
    - Liver tumors in male F344 rats and male and female B6C3F1 mice
    - Thyroid tumors in male and female F344 rats
    - Mesotheliomas of the testis and epididymis in male F344 rats
    - Earlier onset of mononuclear cell leukemia in female F344 rats
    - Clitoral gland tumors in female F344 rats
    - Harderian gland tumors in male and female B6C3F1 mice
    - Reticulum cell sarcomas (type A) (likely histiocytic sarcoma) of the bladder, ovaries, uterus and vagina in female B6C3F1 mice
- Studies of metabolism
    - Carbon-centered free radical formed during reductive metabolism
    - Nitrogen-centered free radical formed during oxidative metabolism
    - Carcinogenic metabolites include formaldehyde, C.I. Basic Red 9, and Michler's ketone (the latter two are products of microbial metabolism and may be produced by intestinal microflora)
    - Multiple additional metabolites with genotoxic activity
- Observations from genotoxicity studies
    - Gentian violet
        - Mutations in *Salmonella typhimurium*, *and E. coli*
        - DNA damage in *B. subtilis*, *E. coli*, and mouse lymphocytes
        - Binding to cell-free calf thymus DNA and synthetic polynucleotides, bacteriophage DNA, bacterial DNA, and chromosomes undergoing mitosis ("mitotic figures") in human oral mucosa tissue
        - CAs in CHO cells, human HeLa cells and cultured lymphocytes, and other mammalian cells
        - Chromosome breakage in CHO and human peripheral blood cells
        - Gene amplification in a SV40-transformed hamster cell line
    - Gentian violet metabolites
        - Pentamethylpararosaniline chloride induces mutations in *Salmonella typhimurium* (four strains), *B. subtilis, E. coli*, and bacteriophage T4D, and binds to calf thymus DNA
        - Leucogentian violet induces mutations in *Salmonella typhimurium*
        - Leuco-pentamethylpararosaniline induces mutations in *Salmonella typhimurium*
        - N,N,N',N'-tetramethylpararosaniline induces mutations in *Salmonella typhimurium* and *E. coli*

        - N,N,N',N''-tetramethylpararosaniline induces mutations in *Salmonella typhimurium* and *E. coli*
        - The carcinogenic metabolites formaldehyde, C.I. Basic Red 9, and Michler's ketone are also genotoxic

- Structure-activity similarities between gentian violet and the seven comparison compounds, two of which are listed as carcinogens under Proposition 65
    - Common target tumor types observed between gentian violet and some of the comparison chemicals are:
        - Hepatocellular tumors (observed for three comparison chemicals)
        - Thyroid tumors (observed for one comparison chemical)
        - Harderian gland tumors (observed for one comparison chemical)
    - Six of the comparison compounds have genotoxic activity.

- Mechanistic findings for gentian violet are associated with the following key characteristics of carcinogens:
    - Is electrophilic and can form electrophilic metabolites
    - Is genotoxic
    - Induces oxidative stress
    - Modulates receptor-mediated effects

7. REFERENCES

Aidoo A, Gao N, Neft RE, Schol HM, Hass BS, Minor TY, et al. 1990. Evaluation of the genotoxicity of gentian violet in bacterial and mammalian cell systems. Teratogenesis Carcinog Mutagen 10:449-462.

Amato Neto V, Pasternak J. 1990. Letter to the editor. Risk of developing cancer in recipients of gentian violet blood. Validity of the interview. [Risco de desenvolver cancer em receptors de sangue violetado. Validade da entrevista]. Journal of the Brazilian Tropical Medicine Society [Revista da Sociedade Brasileira de Medicina Tropical] 23:53.

APVMA. 2014. Crystal (gentian) violet Chemical Review. Australian Pesticides and Veterinary Medicines Authority. Available: https://apvma.gov.au/node/12476.

Arsalan Ali S, Ahmed G, Hamiz-ul-Fawwad S, Waqar SA, Saleem A. 2013. Gentian violet - A blessing in disguise for the developing world. Burns 39:1326-1327.

Beishline K, Azizkhan-Clifford J. 2015. Sp1 and the 'hallmarks of cancer'. FEBS J 282:224-258.

Berrios RL, Arbiser JL. 2011. Effectiveness of gentian violet and similar products commonly used to treat pyodermas. Dermatol Clin 29:69-73.

Berry JM, Huebner E, Butler M. 1996. The crystal violet nuclei staining technique leads to anomalous results in monitoring mammalian cell cultures. Cytotechnology 21:73-80.

Bolderston A, Lloyd NS, Wong RK, Holden L, Robb-Blenderman L, Supportive Care Guidelines Group of Cancer Care Ontario Program in Evidence-Based C. 2006. The prevention and management of acute skin reactions related to radiation therapy: a systematic review and practice guideline. Support Care Cancer 14:802-817.

Bonin AM, Farquharson JB, Baker RS. 1981. Mutagenicity of arylmethane dyes in Salmonella. Mutat Res 89:21-34.

Botts S, Jokinen M, Isaacs KR, Meuten DJ, Tanaka N. 1991. Proliferative lesions of the thyroid and parathyroid glands. In: Guides for Toxicologic Pathology. STP/ARP/AFIP, Washington, DC, 1-12.

Bumpus JA, Brock BJ. 1988. Biodegradation of crystal violet by the white rot fungus Phanerochaete chrysosporium. Appl Environ Microbiol 54:1143-1150.

Caldwell DJ. 1999. Review of mononuclear cell leukemia in F-344 rat bioassays and its significance to human cancer risk: A case study using alkyl phthalates. Regulatory toxicology and pharmacology : RTP 30:45-53.

Chan D, Tarbin JA, Stubbings G, Kay J, Sharman M. 2012. Analysis of incurred crystal violet in Atlantic salmon (Salmo salar L.): comparison between the analysis of crystal

violet as an individual parent and leucocrystal violet and as total crystal violet after oxidation with 2,3-dichloro-5,6-dicyanobenzoquinone. Food Addit Contam Part A-Chem 29:66-72.

Chen CC, Liao HJ, Cheng CY, Yen CY, Chung YC. 2007. Biodegradation of crystal violet by Pseudomonas putida. Biotechnol Lett 29:391-396.

Chen CH, Chang CF, Ho CH, Tsai TL, Liu SM. 2008. Biodegradation of crystal violet by a Shewanella sp NTOU1. Chemosphere 72:1712-1720.

Chi Z, Liu R, Zhao X, Sun Y, Yang B, Gao C. 2009. Study on the genotoxic interaction of methyl violet with calf thymus DNA. Appl Spectrosc 63:1331-1335.

Chung KT, Fulk GE, Andrews AW. 1981. Mutagenicity testing of some commonly used dyes. Appl Environ Microbiol 42:641-648.

Clark AM. 1953. The Mutagenic Activity of Dyes in Drosophila melanogaster. The American Naturalist 87:295-305.

Cooksey CJ. 2017. Quirks of dye nomenclature. 7. Gentian violet and other violets. Biotech Histochem 92:134-140.

Culp SJ, Beland FA, Heflich RH, Benson RW, Blankenship LR, Webb PJ, et al. 2002. Mutagenicity and carcinogenicity in relation to DNA adduct formation in rats fed leucomalachite green. Mutat Res 506-507:55-63.

De Sousa DO, Carneiro SdS, Galvão PM, Irusta RC, Cardoso VM, Luquetti AO. 1989. Cancer risk in recipients of violet-treated blood: validity of the interview. [Risco de desenvolver cancer em receptors de sangue violetado. Validade da entrevista]. Journal of the Brazilian Tropical Medicine Society [Revista da Sociedade Brasileira de Medicina Tropical] 22:108.

Dix DJ, Houck KA, Martin MT, Richard AM, Setzer RW, Kavlock RJ. 2007. The ToxCast program for prioritizing toxicity testing of environmental chemicals. Toxicological sciences : an official journal of the Society of Toxicology 95:5-12.

Docampo R, Moreno SN, Muniz RP, Cruz FS, Mason RP. 1983. Light-enhanced free radical formation and trypanocidal action of gentian violet (crystal violet). Science 220:1292-1295.

Donya SM, Farghaly AA, Abo-Zeid MA, Aly HF, Ali SA, Hamed MA, et al. 2012. Malachite green induces genotoxic effect and biochemical disturbances in mice. Eur Rev Med Pharmacol Sci 16:469-482.

Dunkel VC, Simmon VF. 1980. Mutagenic activity of chemicals previously tested for carcinogenicity in the National Cancer Institute bioassay program. IARC Sci Publ:283-301.

Dunkel VC, Zeiger E, Brusick D, McCoy E, McGregor D, Mortelmans K, et al. 1985. Reproducibility of microbial mutagenicity assays: II. Testing of carcinogens and noncarcinogens in Salmonella typhimurium and Escherichia coli. Environmental Mutagenesis 7:1-19.

Dunn TB. 1954. Normal and pathologic anatomy of the reticular tissue in laboratory mice, with a classification and discussion of neoplasms. J Natl Cancer Inst 14:1281-1433.

ECHA. 2010. Committee for Risk Assessment RAC. Annex 1 Background document to the Opinion proposing harmonised classification and labelling at Community level of leucomalachite green. European Chemicals Agency. Available: https://echa.europa.eu/documents/10162/f468434e-f006-4a34-9cc9-7f652d0b9a9f.

ECHA. 2012. Proposal for identification of a substance as a CMR 1A or 1B, PBT, VPVB or a substance of an equivalent level of concern. Annex XV – Identification of C.I. Basic violet 3 with Michler's ketone or Michler's base ≥0.1% as SVHC. Available: https://echa.europa.eu/documents/10162/13638/svhc_axvrep_c_i_basic_violet_3_pub_en.pdf.

Edwards K. 2016. New Twist on an Old Favorite: Gentian Violet and Methylene Blue Antibacterial Foams. Adv Wound Care (New Rochelle) 5:11-18.

Farid KJ, Kelly K, Roshin S. 2011. Gentian violet 1% solution in the treatment of wounds in the geriatric patient: a retrospective study. Geriatr Nurs 32:85-95.

FDA. 1995. CPG Sec. 578.600 Unapproved Additives for Exported Grains. Available: https://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm074612.htm.

FDA. 2008. Report to Congress. Food and Drug Administration Amendments Act of 2007 Public Law 110-85 Section 1006 – Enhanced Aquaculture and Seafood Inspection. Available: https://www.fda.gov/food/guidanceregulation/guidancedocumentsregulatoryinformation/seafood/ucm150954.htm.

FDA. 2016. FDA Executive Summary. Classification of Wound Dressings Combined with Drugs. Prepared for the Meeting of the General and Plastic Surgery Devices Advisory Panel September 20-21, 2016. Available: https://www.fda.gov/ucm/groups/fdagov-public/@fdagov-afda-adcom/documents/document/ucm518494.pdf [accessed on June 27, 2018].

FDA. 2018. Import Alert 16-131. "Detention Without Physical Examination of Aquacultured, Shrimp, Dace, and Eel from China-Presence of New Animal Drugs and/or Unsafe Food Additives". Available: https://www.accessdata.fda.gov/cms_ia/importalert_33.html.

Frith CH, Ward JM, Chandra M. 1993. The morphology, immunohistochemistry, and incidence of hematopoietic neoplasms in mice and rats. Toxicol Pathol. 21(2):206-218. eng.

Fujita H, Mizuo A, Hiraga K. 1976. Mutagenicity of dyes in the microbial system. Tokyo-toritsu Eisei Kenkyusho Kenkyu Nenpo (Annual report of Tokyo Metropolitan Research Laboratory of Public Health) 27-2:153-158.

Fujita H. 1977. Mutagenicity of dyes in the microbial system. [As cited by Littlefield et al. (1989) and Aidoo et al. (1990)]. In: Chemistry Abstr, Vol. 87, 48460.

Gadelha FR, Hanna PM, Mason RP, Docampo R. 1992. Evidence for free radical formation during horseradish peroxidase-catalyzed N-demethylation of crystal violet. Chem Biol Interact 85:35-48.

GAO. 1980. FDA's regulation of gentian violet appears reasonable. (Report by the Controller General of the United States). US Government Accountability Office. Available: https://www.gao.gov/assets/140/130301.pdf.

Gessner T, Mayer U. 2000. Triarylmethane and Diarylmethane Dyes. In: Ullmann's Encyclopedia of Industrial Chemistry.

Goldstein L. 2015. Nipple Thrush: How to Identify and How to Treat. Midwifery Today Int Midwife:18-19.

Goodman DG, Ward JM, Squire RA, Chu KC, Linhart MS. 1979. Neoplastic and nonneoplastic lesions in aging F344 rats. Toxicol Appl Pharmacol 48:237-248.

Harada T, Enomoto A, Boorman GA, Maronpot RR. 1999. Liver and gallbladder. In: Pathology of the mouse, (Maronpot RR, Boorman GA, Gaul BW, eds). Vienna, IL:Cache River Press, 140-151.

Harrelson WG, Jr., Mason RP. 1982. Microsomal reduction of gentian violet. Evidence for cytochrome P-450-catalyzed free radical formation. Mol Pharmacol 22:239-242.

Harvey SC. 1990. Antimicrobial Drugs: Gentian Violet. In: Remington's Pharmaceutical Sciences, Part 18th (Gennaro A, ed). Easton, PA:Mack Publishing Co, 1171.

Haseman JK, Huff JE, Rao GN, Arnold JE, Boorman GA, McConnell EE. 1985. Neoplasms observed in untreated and corn oil gavage control groups of F344/N rats and (C57BL/6N X C3H/HeN)F1 (B6C3F1) mice. J Natl Cancer Inst 75:975-984.

Haseman JK, Hailey JR, Morris RW. 1998. Spontaneous neoplasm incidences in Fischer 344 rats and B6C3F1 mice in two-year carcinogenicity studies: a National Toxicology Program update. Toxicol Pathol 26:428-441.

Hass BS, Heflich RH, McDonald JJ. Evaluation of the mutagenicity of crystal violet and its metabolites in Salmonella typhimurium and Escherichia coli. (Abstract #95). In:

Proceedings of the The seventeenth annual meeting of the Environmental Mutagen Society, April 9-13, 1986, Vol. 8 Suppl 6, 1-104.

HSDB. 2018. HSDB: Gentian violet (CASRN: 548-62-9).

Huff J. 1992. Applicability to humans of rodent-specific sites of chemical carcinogenicity: Tumors of the forestomach and of the Harderian, preputial, and Zymbal glands induced by benzene. Journal of Occupational Medicine and Toxicology 1:109-141.

IARC. 2006. Volume 88. Formaldehyde, 2-Butoxyethanol and 1-tert-Butoxypropan-2-ol. (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans). Lyon, France. Available: https://monographs.iarc.fr/wp-content/uploads/2018/06/mono88.pdf.

IARC. 2010. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 99. Some Aromatic Amines, Organic Dyes, and Related Exposures. Lyon, France. Available: https://monographs.iarc.fr/wp-content/uploads/2018/06/mono99.pdf.

IARC. 2012. Chemical Agents and Related Occupations. Volume 100 F. A Review of Human Carcinogens. (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans). Lyon, France. Available: https://monographs.iarc.fr/wp-content/uploads/2018/06/mono100F.pdf.

IARC. 2017. IARC Monographs: Instructions to Authors. Originally posted on December 4, 2014. Updated on March 10, 2017. Lyon, France: International Agency for Research on Cancer. Available: https://monographs.iarc.fr/wp-content/uploads/2018/06/Instructions_to_Authors_S4.pdf.

Ishidate M, Jr. 1981. Application of chromosomal aberration tests in vitro to the primary screening for chemicals with carcinogenic and/or genetic hazards. [as cited by ECHA 2010]. Test Courts Cancerog Quo Vadis Symposium:57-79.

Ishmael J, Dugard PH. 2006. A review of perchloroethylene and rat mononuclear cell leukemia. Regulatory toxicology and pharmacology : RTP 45:178-184.

Iyer S, Pham N, Marty M, Sandy M, Solomon G, Zeise L. 2018. An integrative approach for identifying chemicals that affect cancer pathways. (In preparation).

JECFA. 2014. Toxicological evaluatin of certain veterinary drug residues in food. (WHO food additives series). Geneva: The Joint FAO/WHO Expert Committee on Food Additives, World Health Organization. Available: http://www.inchem.org/documents/jecfa/jecmono/v69je01.pdf [accessed on May 2, 2018].

Judson RS, Houck KA, Kavlock RJ, Knudsen TB, Martin MT, Mortensen HM, et al. 2010. In vitro screening of environmental chemicals for targeted testing prioritization: the ToxCast project. Environ Health Perspect 118:485-492.

Kada T, Tutikawa K, Sadaie Y. 1972. In vitro and host-mediated "rec-assay" procedures for screening chemical mutagens; and phloxine, a mutagenic red dye detected. Mutat Res 16:165-174.

Kamber M, Fluckiger-Isler S, Engelhardt G, Jaeckh R, Zeiger E. 2009. Comparison of the Ames II and traditional Ames test responses with respect to mutagenicity, strain specificities, need for metabolism and correlation with rodent carcinogenicity. Mutagenesis 24:359-366.

Kamyab A, McGill E, Firman JD. 2009. The use of hexamethyl-p-rosaniline chloride as an ingredient in poultry feed. World's Poultry Science Journal 65:475-480.

Kavlock R, Chandler K, Houck K, Hunter S, Judson R, Kleinstreuer N, et al. 2012. Update on EPA's ToxCast program: providing high throughput decision support tools for chemical risk management. Chem Res Toxicol 25:1287-1302.

Kinosita R. 1940. Studies on the Cancerogenic Azo and Related Compounds. The Yale Journal of Biology and Medicine 12:287-300.

Kitchin KT, Brown JL. 1994. Dose-response relationship for rat liver DNA damage caused by 49 rodent carcinogens. Toxicology 88:31-49.

Kumar CG, Mongolla P, Basha A, Joseph J, Sarma VU, Kamal A. 2011. Decolorization and biotransformation of triphenylmethane dye, methyl violet, by Aspergillus sp. isolated from Ladakh, India. J Microbiol Biotechnol 21:267-273.

Kvelland I. 1983. The mutagenic effect of methyl violet in phage T4D. Hereditas 98:145-147.

Lafi A, Parry JM, Parry EM. 1986. The effect of Michler's ketone on cell division, chromosome number and structure in cultured Chinese hamster cells. Mutagenesis 1:17-20.

Leaver IH. 1972. Photoreduction of crystal violet in solution and in poly(vinyl alcohol) films. Photochemistry and Photobiology 16:189-196.

Lee JB, Kim HY, Jang YM, Song JY, Woo SM, Park MS, et al. 2010. Determination of malachite green and crystal violet in processed fish products. Food Addit Contam Part A-Chem 27:953-961.

Leifer Z, Kada T, Mandel M, Zeiger E, Stafford R, Rosenkranz HS. 1981. An evaluation of tests using DNA repair-deficient bacteria for predicting genotoxicity and carcinogenicity. A report of the U.S. EPA's Gene-TOX Program. Mutat Res 87:211-297.

Lewis LM, Indig GL. 2002. Effect of dye aggregation on triarylmethane-mediated photoinduced damage of hexokinase and DNA. Journal of Photochemistry and Photobiology B: Biology 67:139-148.

Lian ZR, Wang JT. 2013. Determination of crystal violet in seawater and seafood samples through off-line molecularly imprinted SPE followed by HPLC with diode-array detection. J Sep Sci 36:980-985.

Littlefield NA, Blackwell BN, Hewitt CC, Gaylor DW. 1985. Chronic Toxicity and Carcinogenicity Studies of Gentian-Violet in Mice. Fundam Appl Toxicol 5:902-912.

Littlefield NA, Gaylor DW, Blackwell BN, Allen RR. 1989. Chronic toxicity/carcinogenicity studies of gentian violet in Fischer 344 rats: two-generation exposure. Food Chem Toxicol 27:239-247.

Luquetti AO. 1990. Reply to letter to the editor 23(1):53, 1990: “cancer risk in recipients of violet-treated blood: validity of the interview”. [Resposta à carta ao editor 23(1): 53, 1990: “risco de desenvolver câncer em receptores de Sangue violetado. Validade da entrevista”]. Journal of the Brazilian Tropical Medicine Society [Revista da Sociedade Brasileira de Medicina Tropical] 23:237-238.

Maley AM, Arbiser JL. 2013. Gentian violet: a 19th century drug re-emerges in the 21st century. Exp Dermatol 22:775-780.

Matsui S. 1980. Evaluation of a Bacillus-Subtilis Rec-Assay for the Detection of Mutagens Which May Occur in Water Environments. Water Res 14:1613-1619.

McConnell RF, Westen HH, Ulland BM, Bosland MC, Ward JM. 1992. Proliferative lesions of the testes in rats with selected examples from mice. In: Guides for Toxicologic Pathology. STP/ARP/AFIP, Washington, DC, 1-32.

McDonald JJ, Cerniglia CE. 1984. Biotransformation of gentian violet to leucogentian violet by human, rat, and chicken intestinal microflora. Drug Metabolism and Disposition 12:330-336.

McDonald JJ, North BM, Breeden CR, Lai CC, Roth R. 1984a. Synthesis and disposition of C-14-labelled gentian violet in F344 rats and B6C3F1 mice. Food Chem Toxicol 22:331-336.

McDonald JJ, Breeden CR, North BM, Roth RW. 1984b. Species and strain comparison of the metabolism of gentian violet by liver microsomes. Journal of Agricultural and Food Chemistry 32:596-600.

Mirsalis JC, Tyson CK, Steinmetz KL, Loh EK, Hamilton CM, Bakke JP, et al. 1989. Measurement of unscheduled DNA synthesis and S-phase synthesis in rodent hepatocytes following in vivo treatment: testing of 24 compounds. Environ Mol Mutagen 14:155-164.

Mitchell AD, Myhr BC, Rudd CJ, Caspary WJ, Dunkel VC. 1988. Evaluation of the L5178Y mouse lymphoma cell mutagenesis assay: methods used and chemicals evaluated. Environ Mol Mutagen 12 Suppl 13:1-18.

Mittelstaedt RA, Mei N, Webb PJ, Shaddock JG, Dobrovolsky VN, McGarrity LJ, et al. 2004. Genotoxicity of malachite green and leucomalachite green in female Big Blue B6C3F1 mice. Mutat Res 561:127-138.

Mortelmans K, Haworth S, Lawlor T, Speck W, Tainer B, Zeiger E. 1986. Salmonella mutagenicity tests: II. Results from the testing of 270 chemicals. Environ Mutagen 8 Suppl 7:1-119.

Motiwale G, Jaiswal S, Vikey A, Motiwale T, Bagulkar B, Bhat A, et al. 2016. Evaluation of efficacy of 1% crystal violet & nuclear fast red stain compared to haematoxyline & eosin stain for assessing mitotic figures in oral premalignant and malignant lesions. Journal of Experimental Therapeutics and Oncology 11:165-170.

Muller W, Gautier F. 1975. Interactions of heteroaromatic compounds with nucleic acids. A - T-specific non-intercalating DNA ligands. Eur J Biochem 54:385-394.

Myhr BC, Caspary WJ. 1988. Evaluation of the L5178Y mouse lymphoma cell mutagenesis assay: intralaboratory results for sixty-three coded chemicals tested at Litton Bionetics, Inc. Environ Mol Mutagen 12 Suppl 13:103-194.

NCI. 1979. Bioassay of Michler's Ketone for Possible Carcinogenicity. CAS No. 90-94-8. (Carcinogenesis Technical Report Series No 181). National Cancer Institute. Available: https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr181.pdf.

NCTR. 1983. NCTR technical report, experiment 304. Chronic toxicity and carcinogenicity studies of gentian violet in mice. Jefferson, Arkansas: National Center for Toxicological Research.

NCTR. 1985. NCTR technical report for experiment 6040. Metabolism of gentian violet in chickens. Jefferson, Arkansas: National Center for Toxicological Research.

NCTR. 1988. NCTR technical report for experiment 388. Chronic toxicity and carcinogenicity studies of gentian violet in Fischer 344 rats. Jefferson, Arkansas: National Center for Toxicological Research.

NCTR. 1989. NCTR technical report for experiments 302, 303. Metabolism of gentian violet in Fischer 344 rats and B6C3F1 mice. Jefferson, Arkansas: National Center for Toxicological Research.

NTP. 1986. NTP Toxicology and Carcinogenesis Studies of C.I. Basic Red 9 Monohydrochloride (Pararosaniline) (CAS No. 569-61-9) In F344/N Rats and B6C3F1 Mice (Feed Studies). (Natl Toxicol Program Tech Rep Ser). 0888-8051 (Print) 0888-8051 (Linking). Available: https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr285.pdf.

NTP. 2004. Technical Report Series, Number 71, NIH Publication No. 04-4416. NTP technical report on the toxicity studies of malachite green chloride and leucomalachite green (CAS Nos. 569-64-2 and 129-73-7) administered in feed to F344/N rats and B6C3F1 mice. Available: https://ntp.niehs.nih.gov/ntp/htdocs/st_rpts/tox071.pdf.

NTP. 2005. NTP technical report on the toxicity and carcinogenesis studies of malachite green chloride and leucomalachite green (CAS Nos. 569-64-2 and 129-73-7) in F344/N rats and B6C3F1 mice (feed studies). Research Triangle Park, NC. Available: https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr527.pdf.

NTP. 2018. Michler's ketone (90-94-8). Chemical Effects in Biological Systems (CEBS). Research Triangle Park, NC (USA).

Olentine JCG, Cross DL, Burrows PM. 1980. 14C-Gentian Violet Residues in Tissues of Broiler Breeders. Poultry Science 59:500-505.

Ozbay G, Babu BK, Chen G. 2013. Prevalence of Veterinary Drug Residues and Heavy Metals in Catfish Nuggets. Journal of Food Processing & Technology S11.

Parodi S, Santi L, Russo P, Albini A, Vecchio D, Pala M, et al. 1982. DNA damage induced by auramine O in liver, kidney, and bone marrow of rats and mice, and in a human cell line (alkaline elution assay and SCE induction). J Toxicol Environ Health 9:941-952.

Passmore F. 1890. Ketone Compounds. In: Pharmaceutical Journal and Transactions, Vol. 21. London, England:J & A Churchill, 548.

Popescu A, Doyle RJ. 1996. The Gram stain after more than a century. Biotech Histochem 71:145-151.

Ramirez LE, Lages-Silva E, Pianetti GM, Rabelo RM, Bordin JO, Moraes-Souza H. 1995. Prevention of transfusion-associated Chagas' disease by sterilization of Trypanosoma cruzi-infected blood with gentian violet, ascorbic acid, and light. Transfusion 35:226-230.

Reszka K, Cruz FS, Docampo R. 1986. Photosensitization by the trypanocidal agent crystal violet. Type I versus type II reactions. Chem Biol Interact 58:161-172.

Rudmann D, Cardiff R, Chouinard L, Goodman D, Küttler K, Marxfeld H, et al. 2012. Proliferative and Nonproliferative Lesions of the Rat and Mouse Mammary, Zymbal’s, Preputial, and Clitoral Glands. Toxicologic Pathology 40:7S-39S.

Schaeppi HU. 1955. Experiments with cancer- and leukemia producing characteristics of methyl violet. Zeitschrift für Unfallmedizin und Berufskrankheiten Revue de médecine des accidents et des maladies professionelles 48:48-68.

Schmunis GA. 1999. Prevention of transfusional Trypanosoma cruzi infection in Latin America. Mem Inst Oswaldo Cruz 94 Suppl 1:93-101.

Schuetze A, Heberer T, Juergensen S. 2008. Occurrence of residues of the veterinary drug malachite green in eels caught downstream from municipal sewage treatment plants. Chemosphere 72:1664-1670.

Scribner JD, Koponen G, Fisk SR, Woodworth B. 1980. Binding of the dye intermediates Michler's ketone and methane base to rat liver nucleic acids and lack of mutagenicity in Salmonella typhimurium. Cancer Lett 9:117-121.

Shahin MM, Von Borstel RC. 1978. Comparisons of mutation induction in reversion systems of Saccharomyces cerevisiae and Salmonella typhimurium. Mutation Research/Environmental Mutagenesis and Related Subjects 53:1-10.

Sheldon WG. 1994. Tumours of the Harderian gland. In: Pathology of tumours in laboratory animals Volume II Tumours of the mouse IARC Scientific Publications No 111, (Turusov VS, Mohr U, eds). Lyon, France, 100-113.

Sheng Z, Chen L. 2017. Switch-on fluorescent strategy based on crystal violet-functionalized CdTe quantum dots for detecting L-cysteine and glutathione in water and urine. Anal Bioanal Chem 409:6081-6090.

Smith MT, Guyton KZ, Gibbons CF, Fritz JM, Portier CJ, Rusyn I, et al. 2016. Key Characteristics of Carcinogens as a Basis for Organizing Data on Mechanisms of Carcinogenesis. Environ Health Perspect 124:713-721.

Speidel D. 2015. The role of DNA damage responses in p53 biology. Arch Toxicol 89:501-517.

Stromberg PC, Vogtsberger LM. 1983. Pathology of the mononuclear cell leukemia of Fischer rats. I. Morphologic studies. Veterinary pathology 20:698-708.

Stromberg PC. 1985. Large granular lymphocyte leukemia in F344 rats. Model for human T gamma lymphoma, malignant histiocytosis, and T-cell chronic lymphocytic leukemia. The American journal of pathology 119:517-519.

Struck RF, Shih TW, Johnston TP, Kirk MC, Hill DL. 1981. Metabolism and macromolecular binding of the carcinogen Michler's ketone in rats. Xenobiotica 11:569-567.

Sugimura T, Sato S, Nagao M, Yahagi T, Matsushima T, Seino Y, et al., eds. 1976. Overlapping of carcinogens and mutagens. Baltimore, MD:University Park Press.

Sun Q, Luo Y, Yang X, Xiang P, Shen M. 2016. Detection and identification of dyes in blue writing inks by LC-DAD-orbitrap MS. Forensic Sci Int 261:71-81.

Suzuki T, Hara T, Kitagawa Y, Yamaguchi T. 2016. Magnified endoscopic observation of early colorectal cancer by linked color imaging with crystal violet staining (with video). Gastrointest Endosc 84:726-729.

Tennant RW, Stasiewicz S, Spalding JW. 1986. Comparison of multiple parameters of rodent carcinogenicity and in vitro genetic toxicity. Environ Mutagen 8:205-227.

Thetford D. 2013. Triphenylmethane and Related Dyes (updated by staff). In: Kirk-Othmer Encyclopedia of Chemical Technology:John Wiley & Sons, Inc.

Thomas J, Haseman JK, Goodman JI, Ward JM, Loughran TP, Jr., Spencer PJ. 2007. A review of large granular lymphocytic leukemia in Fischer 344 rats as an initial step toward evaluating the implication of the endpoint to human cancer risk assessment. Toxicological sciences : an official journal of the Society of Toxicology 99:3-19.

Thompson HC, Rushing LG, Gehring T, Lochmann R. 1999. Persistence of gentian violet and leucogentian violet in channel catfish (ictalurus punctatus) muscle after water-borne exposure. J Chromatogr B 723:287-291.

US EPA. 2012. Toxicological review of tetrachloroethylene (Perchloroethylene) (CAS No. 127-18-4). In support of summary information on the Integrated Risk Information System (IRIS). Washington, D.C.: U.S. Environmental Protection Agency.

US Pharmacopeia. 2014. Gentian Violet Safety Data Sheet, Version #03.

van Dam H, Castellazzi M. 2001. Distinct roles of Jun : Fos and Jun : ATF dimers in oncogenesis. Oncogene 20:2453.

Van der Zee J, Duling DR, Mason RP, Eling TE. 1989. The oxidation of N-substituted aromatic amines by horseradish peroxidase. J Biol Chem 264:19828-19836.

Vilaseca GC, Cerisola JA, Olarte JA, Zothner A. 1966. The use of crystal violet in the prevention of the transfusional transmission of Chagas-Mazza disease (South American trypanosomiasis). Vox Sang 11:711-716.

Wakelin LP, Adams A, Hunter C, Waring MJ. 1981. Interaction of crystal violet with nucleic acids. Biochemistry 20:5779-5787.

Woo KY, Heil J. 2017. A prospective evaluation of methylene blue and gentian violet dressing for management of chronic wounds with local infection. Int Wound J 14:1029-1035.

Yamaguchi T. 1988. Adsorption of Carcinogenic and/or Mutagenic Pigments on DNA-binding Sepharose. Agricultural and Biological Chemistry 52:845-847.

Yatome C, Ogawa T, Matsui M. 1991. Degradation of crystal violet by bacillus subtilis. Journal of Environmental Science and Health Part A: Environmental Science and Engineering and Toxicology 26:75-87.

Yatome C, Yamada S, Ogawa T, Matsui M. 1993. Degradation of Crystal violet by Nocardia corallina. Applied Microbiology and Biotechnology 38:565-569.

Yoshizawa K. 2018. Chapter 20 - Specialized Sebaceous Glands—Zymbal's Gland, Preputial Gland, Clitoral Gland, and Perianal Gland. In: Boorman's Pathology of the Rat (Second Edition), (Suttie AW, ed). Boston:Academic Press, 347-365.

Zhang Z, Zhou K, Bu YQ, Shan ZJ, Liu JF, Wu XY, et al. 2012. Determination of malachite green and crystal violet in environmental water using temperature-controlled ionic liquid dispersive liquid-liquid microextraction coupled with high performance liquid chromatography. Anal Methods 4:429-433.

Zimina TA, Pavlenko VV. 1990. Toxicogenetic effects of azo- and arylmethane dyes. Genetika 26:2246-2249.

Appendix A. Literature Search Strategies on the Carcinogenicity of Gentian Violet

General searches of the literature on the carcinogenicity of gentian violet were conducted to identify peer-reviewed open-source and proprietary journal articles, print and digital books, reports, and gray literature that potentially reported relevant toxicological and epidemiological information on the carcinogenicity of this chemical. The search sought to identify all literature relevant to the assessment of evidence on the carcinogenicity of gentian violet.

Search Process

Relevant subject terms were entered into the PubMed Search Builder to execute a search.

The following is a typical chemical search strategy used to search PubMed:

("*chemical name*" [MeSH] OR "CAS *registry number*"[RN]) AND ("bioassay"[MeSH] OR "carcinogenicity"[MeSH] OR "cancer"[MeSH] OR "tumor"[MeSH]) OR "neoplasm"[MeSH]) OR "genotoxicity"[MeSH]) OR "mutagenicity"[MeSH]) OR "metabolism"[MeSH]) OR "absorption"[MeSH]) OR "pharmacokinetics"[MeSH]) OR "structure activity relationship"[MeSH])

ChemID*plus* (https://chem.nlm.nih.gov/chemidplus), a ToxNet database, was used to search for synonyms of gentian violet.

In PubMed, MeSH (Medical Subject Headings) terms at the top of hierarchical lists of subject headings are automatically "exploded" in a search to retrieve citations with more specific MeSH terms. For example, the heading "carcinogenicity" includes broad conditions that are related to cancer induction in animals and humans.

Additional databases and other data sources listed below were then searched. The search strategies were tailored according to the search features unique to each database and data source. Web of Science, for example, was searched by entering chemical terms and refining the search by applying Web of Science categories Toxicology and/or Public, Environmental and Occupational Health. The search term used includes either the CAS registry number or the chemical name and its available synonyms. Sometimes other databases and data sources not listed here were searched as needed.

Relevant literature was also identified from citations in individual articles.

Data Sources

The following is a list of the major data sources that were searched to find information on gentian violet. The list was recommended by the National Toxicology Program (NTP) handbook for preparing Report on Carcinogens (RoC) monographs (NTP, 2015) and modified by OEHHA.

Biomedical literature databases

- PubMed (National Library of Medicine) (https://www.ncbi.nlm.nih.gov/pubmed)
- TOXNET (National Library of Medicine): Toxicology Literature Online (TOXLINE) (https://toxnet.nlm.nih.gov)
- Scopus (https://www.scopus.com/search/form.uri?display=basic)
- Embase (https://www.elsevier.com/solutions/embase-biomedical-research)
- Web of Science® (Thomson-Reuters, Inc.) (https://clarivate.com/products/web-of-science)

Authoritative reviews and reports

- International Agency for Research on Cancer (IARC) Monographs (https://monographs.iarc.fr/monographs-and-supplements-available-online)
- National Toxicology Program (NTP) publications, including, but not limited to, technical reports, nominations for toxicological evaluation documents, Report on Carcinogens (RoC) monographs, RoC background documents or monographs, and NTP Office of Health Assessment and Translation (OHAT) monographs (https://ntp.niehs.nih.gov)
- US Environmental Protection Agency (EPA) Integrated Risk Information System (IRIS) (https://www.epa.gov/iris)
- Agency for Toxic Substances and Disease Registry (ATSDR) Toxicological Profiles (https://www.atsdr.cdc.gov/toxprofiles/index.asp)
- European Chemicals Agency Risk Assessments (https://echa.europa.eu)
- Health Canada Environmental Health Assessments (https://www.canada.ca/en/health-canada.html)
- New York State Department of Health — Health Topics A to Z (https://www.health.ny.gov/healthaz)
- National Academy of Sciences reports and publications (https://www.nationalacademies.org/publications)

- World Health Organization (WHO)/United Nations Environment Programme (UNEP) International Programme on Chemical Safety (IPCS) INCHEM-related documents (http://www.inchem.org)

Other Databases or web resources

- TOXNET: Genetic Toxicology Data Bank (GENE-TOX) (https://toxnet.nlm.nih.gov/newtoxnet/genetox.htm), Carcinogenic Potency Database (CPDB) (https://toxnet.nlm.nih.gov/newtoxnet/cpdb.htm)
- Comparative Toxicogenomics Database (CTD) (http://ctdbase.org)
- US EPA iCSS Dashboard v2 (ToxCast Phase II data, (https://actor.epa.gov/dashboard2/)
- European Food Safety Authority (http://www.efsa.europa.eu/en/publications.htm)
- International Labour Organization (http://www.ilo.org/global/publications/lang--en/index.htm)
- International Uniform Chemical Information Database (https://iuclid6.echa.europa.eu)
- National Institute for Occupational Safety and Health (NIOSH) Publications (https://www2.cdc.gov/nioshtic-2/)
- United Nations Environment Programme (https://www.unenvironment.org)
- PubChem BioAssay (National Library of Medicine) (https://www.ncbi.nlm.nih.gov/pcassay)
- Google search engine (https://www.google.com)

The following search strings, in whole or in part, were applied to the databases listed above, when applicable: ("gentian violet" [MeSH] OR "Hexamethylpararosaniline chloride" OR "548-62-9 [RN]") AND ("Neoplasms" [MeSH] OR "Cancer" [MeSH] OR "Mutation" [MeSH] AND "Toxicity" [MeSH] OR "Mechanism" [MeSH] OR "Metabolism" [MeSH]).

In summary, more than 420 references, including government reports, peer-reviewed journal articles and books, were identified through these search strategies. Among these, 139 references were cited in this document.

Appendix B. Genotoxicity Section of JECFA (2014)

OEHHA has attached the relevant pages from JECFA (2014) that discuss the genotoxicity of gentian violet.

Table 13. Incidence of non-neoplastic lesions in Fischer 344 rats fed gentian violet in the diet for 24 months

Site and type of lesion	Incidence of lesion[a]			
	0 mg/kg feed	100 mg/kg feed	300 mg/kg feed	600 mg/kg feed
Males				
Liver				
Clear cell foci	6/179 (3%)	5/90 (6%)	5/88 (6%)	8/89 (9%)
Eosinophilic foci	7/179 (4%)	5/90 (6%)	20/88 (23%)	33/89 (37%)
Mixed foci	32/179 (18%)	26/90 (29%)	28/88 (26%)	47/89 (53%)
Regeneration	7/179 (4%)	11/90 (12%)	21/88 (24%)	15/89 (17%)
Centrilobular necrosis	5/179 (3%)	4/90 (4%)	8/88 (9%)	11/89 (12%)
Thyroid gland				
Follicular cysts	18/163 (11%)	7/84 (8%)	9/74 (12%)	17/97 (22%)
Spleen				
Red pulp hyperplasia	11/175 (6%)	7/88 (8%)	3/87 (3%)	15/86 (17%)
Lymph node	8/168 (5%)	9/86 (10%)	5/84 (6%)	11/81 (14%)
Females				
Liver				
Clear cell foci	1/170 (1%)	1/90 (1%)	3/84 (4%)	1/87 (1%)
Eosinophilic foci	0/170 (0%)	0/90 (0%)	6/84 (7%)	10/87 (11%)
Mixed cell foci	29/170 (17%)	32/90 (36%)	39/84 (46%)	30/87 (34%)
Regeneration	4/170 (2%)	9/90 (10%)	20/84 (24%)	18/87 (21%)
Centrilobular necrosis	7/170 (4%)	8/90 (9%)	6/84 (7%)	20/87 (23%)
Thyroid gland				
Follicular cysts	8/159 (5%)	9/83 (11%)	8/76 (11%)	7/77 (9%)

[a] Incidence is expressed as the number of rats with the identified non-neoplastic lesion divided by the number of rats at risk. Values in parentheses represent the incidence of the non-neoplastic lesions expressed as a percentage of the number of rats surviving.

Source: Littlefield et al. (1989)

JECFA's summary of gentoxocity starts here →

Levin, Lovely & Klekowski (1982) studied the effect of light (plates containing gentian violet were irradiated at 23°C with a Sylvania tungsten/halogen lamp for 3 minutes at 20 cm) on the genotoxicity of gentian violet. In the Rosenkranz assay, a genotoxic effect was observed under conditions of dark and was enhanced by the irradiation. Harrelson & Mason (1982) reported that in the presence of NADPH and light, gentian violet was photoreduced to the same triarylmethyl free radical that is formed by enzymatic reduction. The presence of S9 had no effect on the genotoxicity of gentian violet. However, in the Ames test, where no mutagenic activity was observed but gentian violet was sufficiently toxic to sterilize the plate under conditions of dark incubation, the presence of S9 (active or thermally deactivated) virtually eliminated the toxicity of gentian violet under dark incubation and greatly decreased its toxicity under light conditions.

Table 14. Results of tests for genotoxicity and mutagenicity with gentian violet

Test system	Test object	Concentration	Results	Reference
In vitro				
Rosenkranz repairable DNA assay	*Escherichia coli* DNA polymerase-deficient strain	Not stated	Positive	Rosenkranz & Carr (1971)
Cytogenetic toxicity	CHO cells, human lymphocytes and HeLa and L cells, as well as *Peromyscus eremicus* and Indian Muntjac cell lines	0, 0.5 or 5 µg/mL	Positive (mitotic poison and clastogen)	Au et al. (1978)
Ames test[a]	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0, 1, 2 or 4 µg/plate	Negative, TA1535 equivocal	Shahin & von Borstel (1978)
	Saccharomyces cerevisiae XV185-14C	0, 2, 4, 6 or 8 µg/plate	Negative	
Chromosome breakage	CHO cells	0 or 10 µmol/L	Positive	Au & Hsu (1979)
Ames test[a]	*S. typhimurium* TA98, TA100, TA1535, TA1537	0, 0.1–50 µg	Negative, bactericidal ≥ 10 µg (no activation)	Au et al. (1979)
Rosenkranz repairable DNA assay[b]	*E. coli* W3110 pol A^+, mutant p3478 pol $A^=$	1, 10, 25 or 100 µg/plate	Positive	
Chromosome breakage[c]	CHO cells	5, 10 or 20 µg/mL	Positive, no activation	
Ames test[b]	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0, 0.1, 0.32, 1 or 3.2 µg/plate	Negative except for TA1535 only at 0.32 µg, and no activation	Bonin, Farquharson & Baker (1981)

OEHHA's comment:
Shalin & von Borstel (1978) states that the tested substance was "methyl violet (crystal violet; gentian violet)"; however, Figure 1 of the publication shows the chemical structure of the test substance, which is pentamethylpararosaniline chloride. Pentamethylpararosaniline chloride may be present in commercial gentian violet preparations at low levels (i.e., typically <4%). The rest of the publication referred to the chemical as "methyl violet", a common name for pentamethylpararosaniline chloride.

Table 14 (continued)

Test system	Test object	Concentration	Results	Reference
Chromatid breakage	Human peripheral blood cells	20 µg/mL	Positive	Hsu, Cherry & Pathak (1982)
Ames test[b]	*S. typhimurium* TA98, TA100, TA1537	0, 1, 5, 10 or 50 µg/plate	Negative[c]	Levin, Lovely & Klekowski (1982)
Rosenkranz repairable DNA assay[b]	*E. coli* W3110 pol A^+, mutant p3478 pol $A^=$	0.1, 0.5, 1, 5, 7 or 10 µg/plate	Positive	
Ames test[b]	*S. typhimurium* TA1535	0, 0.025, 0.05, 0.1 or 0.5 µg/plate	Negative	Thomas & MacPhee (1984)
	E. coli DG1669	0, 25, 50, 75 or 100 µg/plate	Positive[d,e]	
Chromosome damage	Human lymphocytes[f,g]	1 µg/mL	Positive	Krishnaja & Sharma (1995)
Ames test	*S. typhimurium* TA97, TA98, TA100 *E. coli* WP2s	1–50 µg of metabolites/ plate for *Salmonella* 5 µmol/L concentration for *E. coli*	Negative (metabolites) in *Salmonella* test Positive in *E. coli* test, maybe metabolites positive as well	Hass, Heflich & McDonald (1986)
Ames test	*S. typhimurium* TA97, TA98, TA100, TA104	0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 µg/plate with or without S9	Positive in TA97 with and without S9, positive in TA104 with S9, negative in others	Aidoo et al. (1990)
Mammalian cell mutagenicity	CHO-K1-BH_4 and CHO-AS52 cells	0–1.5 µg/mL with or without S9	Negative for CHO-K1-BH_4 cells, equivocal results for CHO-AS52 cells	

Table 14 (continued)

Test system	Test object	Concentration	Results	Reference
Lymphocyte DNA damage assay	B6C3F1 mouse	0, 0.2, 0.4, 0.6, 0.8 or 1.0 µg/mL	Positive for DNA damage	
Gene amplification test	SV40-transformed Chinese hamster cell line C060	0.02, 0.05 or 0.125 µg/mL	Dose-related weak SV40 DNA amplification	
In vivo				
Chicken embryo assay	Chicken embryos	0.5, 2, 5, 10, 20, 100, 1 000 or 2 000 µg/embryo	Toxicity ≥ 20 µg; no increase in sister chromatid exchange	Au et al. (1979)
Bone marrow assay	Mouse	Drinking-water 20 or 40 µg/mL for 4 weeks, calculated to be 4 and 8 mg/kg bw per day, respectively	No chromosome damage, decreased mitotic index	
Lymphocyte DNA damage assay	B6C3F1 mouse	Animals treated with 0, 2, 4 or 6 mg/kg bw as a single dose	Negative for DNA damage	Aidoo et al. (1990)

bw: body weight; CHO: Chinese hamster ovary; DNA: deoxyribonucleic acid; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Without metabolic activation.

[b] With and without metabolic activation.

[c] At concentrations above 5.0 µg/plate, gentian violet was sufficiently toxic to sterilize the plates without S9 under conditions of dark incubation. The presence of S9 virtually eliminated the toxicity of gentian violet in the dark and greatly decreased the toxicity in the light.

[d] At gentian violet concentrations of 75 and 100 µg/plate with no S9, a large number of the cells were killed.

[e] With S9, all concentrations of gentian violet resulted in similar numbers of mutants.

[f] Cultured blood lymphocytes from β-thalassaemia traits and healthy individuals.

[g] The incidence of chromatid aberrations was not statistically different between normal and β-thalassaemia trait blood samples.

Thomas & MacPhee (1984) pointed out that all of the strains used by Au et al. (1979) and Bonin, Farquharson & Baker (1981) carried *rfa* mutations and were exceptionally sensitive to the toxic effects of gentian violet and thus not the most suitable strains to assess the mutagenicity of gentian violet, other than at very low dose levels. These authors reported negative results with *Salmonella typhimurium* strain TA1535 in the Ames assay using low doses (0.025–0.5 µg/plate) of gentian violet because of the toxic effects of gentian violet and thus disagreed with the positive results by Bonin, Farquharson & Baker (1981). However, the authors concluded that the positive results with DG1669 (an *Escherichia coli* K12 derivative that carries the *lac*Z(ICR24) frameshift marker and is DNA repair proficient) indicated that gentian violet is a direct-acting mutagen causing frameshift mutations in repair-proficient bacteria. Dose levels of 75 and 100 µg/plate were toxic when S9 was omitted, but not when S9 was present in the incubation mixture.

Aidoo et al. (1990) re-evaluated the genotoxicity of gentian violet (> 96% gentian violet, with the remainder being mainly methyl violet) by conducting mutagenesis and DNA damage experiments in both bacterial and mammalian cell systems. Mutagenicity of gentian violet in *Salmonella* was strain specific; it was mutagenic in TA97 and TA104 strains, but not in TA98 and TA100 strains. S9 tended to increase its mutagenicity. *N,N,N′,N″*-Tetramethylpararosaniline, a metabolite of gentian violet, was a weak mutagen in *Salmonella*. Gentian violet was not mutagenic in Chinese hamster ovary (CHO) cell line CHO-K1-BH_4, but equivocal results were obtained with CHO-AS52 cells. Gentian violet produced DNA damage in B6C3F1 mouse lymphocytes in vitro, but not in vivo. However, the dose levels used in these in vivo tests were much lower than those used in the carcinogenicity studies. The authors concluded that gentian violet is a point mutagen in bacteria and may be carcinogenic in mammalian cells by its clastogenic activity.

JECFA's summary of genotoxicity ends here →

Gentian violet was found to break chromosomes in cultures of CHO cells (Au et al., 1978; Au & Hsu, 1979), human lymphocytes, HeLa and L cells and fibroblastic cell lines (Au et al., 1978).

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

(i) Rats

In a three-generation reproductive toxicity study, gentian violet (99% gentian violet, 1% methyl violet) was administered in feed to Fischer 344 rats at a dose of 0, 100, 300 or 600 mg/kg (equivalent to 0, 5, 15 and 30 mg/kg bw per day, respectively). F_0 animals of both sexes were randomly allocated to treatment groups and fed the medicated ration for at least 80 days. Males and females of the same dose group were then caged together for 14 days for mating, after which males were returned to their own cages. Pups from this mating (F_{1a} generation) were used for a separate study. Following this, 90 rats of each sex for the control group and 45 rats of each sex for each treatment group were selected to continue in this study. F_0 animals

There were few data available on the acute and short-term toxicity of gentian violet, but the reported range of LD_{50}s, from 100 to 800 mg/kg bw, shows that it is of moderate acute oral toxicity. The most common sign of toxicity was lethargy, followed by anorexia and, in some animals, diarrhoea, excessive thirst, emesis and weight loss. In 90-day studies in rats and dogs, the only reported signs were slight body weight loss and increased liver weight, respectively.

In a 24-month study, gentian violet was given to mice in the feed at a concentration of 0, 100, 300 or 600 mg/kg (equal to 0, 10.7–14.3, 32.1–35.7 and 64.3 mg/kg bw per day for males and 0, 14.3, 35.7–39.3 and 71.4 mg/kg bw per day for females, respectively). Few dose-related non-neoplastic lesions were reported, but there were statistically significant dose-related increases in erythropoiesis in the spleen and atrophy of the ovaries in females at 24 months. The LOAEL for non-carcinogenic effects was 14.3 mg/kg bw per day, the lowest dose tested. Significant, dose-related increases in neoplastic lesions were observed in both sexes, with the female mice being more sensitive. Hepatocellular adenomas and carcinomas were the most common lesions, with significant, dose-related increases found at 24 months in males and at both 18 and 24 months in females. Mortality due to liver neoplasms showed positive trends in both males and females, and there was a dose-related decrease in the time for the onset of liver neoplasms. The females also showed statistically significant dose-related increases in adenoma of the Harderian gland and in type A reticulum cell sarcoma in the urinary bladder, uterus, ovaries and vagina. The data clearly indicate that gentian violet is a multisite carcinogen in the mouse.

In a long-term study of toxicity, rats were exposed to gentian violet in the feed at a concentration of 0, 100, 300 or 600 mg/kg (equal to approximately 0, 30, 80 and 160 mg/kg bw per day for males and 0, 40, 100 and 200 mg/kg bw per day for females, respectively). Gentian violet exposure of these animals was achieved by dosing the parents of the study animals prior to and during mating, with the same dose fed to the offspring from weaning up to 24 months of age. There was a statistically significant increase in liver regeneration in all dose groups and statistically significant dose-related increases in eosinophilic foci in the liver in both sexes in both the mid- and high-dose groups. For liver centrilobular necrosis, there was a dose-related increase, but statistical significance was seen only in the 300 mg/kg feed group in males and in the 600 mg/kg feed group in females. As in mice, female rats appeared to be more sensitive than males. The incidence of thyroid adenocarcinoma was increased in males, with statistical significance at the top dose only at 24 months. Females showed a statistically significant dose–response relationship for thyroid adenocarcinoma at 24 months. The incidence of hepatocellular adenomas showed a small but significant dose–response relationship in males and a significant increase in females at 300 mg/kg feed, but not at other doses. The data indicate a carcinogenic response to gentian violet in rats, although much weaker than the response in mice.

JECFA's evaluation/ conclusion on genotoxicity starts here →

The data show that gentian violet binds to DNA, and this, together with the cellular toxicity of gentian violet, complicates both the testing of gentian violet in vitro and the interpretation of the results. The results are somewhat varied in *Salmonella typhimurium*, with positive responses in some strains but not in others. Gentian violet was clastogenic in vitro and positive in indicator tests for DNA damage. There are few in vivo tests on gentian violet. A single in vivo test for clastogenicity (mouse

bone marrow assay) showed no evidence of clastogenic activity, but the Committee noted that the gentian violet was given via the drinking-water at lower doses (4 and 8 mg/kg bw per day) than those used in the mouse cancer bioassay (ranging from 10 to 70 mg/kg bw per day). Similarly, the other in vivo test on DNA damage in mouse lymphocytes using single intravenous doses up to 6 mg/kg bw showed no effect. The Committee concluded that, overall, the data show that gentian violet is genotoxic.

JECFA's evaluation/ conclusion on genotoxicity ends here

In view of the carcinogenicity of gentian violet in the mouse and rat and evidence showing genotoxicity in a number of tests, the Committee concluded that gentian violet should be considered a carcinogen acting by a genotoxic mode of action.

In a multigeneration reproductive toxicity study, rats were given gentian violet in the feed at a concentration of 0, 100, 300 or 600 mg/kg (equivalent to 0, 5, 15 and 30 mg/kg bw per day, respectively) over three generations. There were significant reductions in body weight in the top dose group in all generations. The NOAEL for parental toxicity was 15 mg/kg bw per day. In the F_{3a} generation, examined for histopathological effects, a dose-related trend for focal dilatation of the renal cortex and tubules, a statistically significant dose-related trend for necrosis of the thymus and an inverse dose–response relationship for red pulp haematopoietic cell proliferation of the spleen were seen. The effects in the F_{3a} generation were present in all dose groups, and a NOAEL for offspring toxicity could not be determined. Gentian violet had no effect on the number of pups per litter, fertility index, pup survival, sex ratio or number of stillborn animals. The NOAEL for reproductive toxicity was 30 mg/kg bw per day, the highest dose tested.

Two developmental toxicity studies were conducted in rats. In the first study, CD rats were given gentian violet at 0, 2.5, 5 or 10 mg/kg bw per day by oral gavage on days 6–15 of gestation. In the second study, the three-generation study in Fischer 344 rats described above, the F_{3b} generation was examined for teratogenic effects. In that study, gentian violet was given in the feed at a concentration of 0, 100, 300 or 600 mg/kg (equivalent to 0, 5, 15 and 30 mg/kg bw per day, respectively). CD rats appeared to be more sensitive than Fischer 344 rats to the toxicity of gentian violet, with dose-related reductions in maternal weight gain at 5 and 10 mg/kg bw per day and increased clinical signs of toxicity, significant at 10 mg/kg bw per day and limited at 5 mg/kg bw per day (maternal toxicity NOAEL of 2.5 mg/kg bw per day). In Fischer 344 rats, reduction in body weight was seen only at 30 mg/kg bw per day and not at lower doses of 5 and 15 mg/kg bw per day (maternal toxicity NOAEL of 15 mg/kg bw per day). It was also noted that malformations (hydroureter, hydronephrosis and short ribs) were seen only in the CD rats. Effects on the fetus were seen only at doses that also caused maternal toxicity. The NOAEL for embryo and fetal toxicity in CD rats was 5 mg/kg bw per day.

In a developmental toxicity study, rabbits were given gentian violet at 0, 0.5, 1 or 2 mg/kg bw per day by oral gavage on days 6–19 of gestation. Maternal mortality was increased in a dose-related manner, and maternal body weight gain was decreased in all treated groups compared with controls. Fetal weights were significantly reduced in all treated groups compared with controls. There was no evidence of teratogenic effects. Owing to the presence of maternal toxicity and significantly reduced fetal weights in all dosed groups, NOAELs could not be identified for maternal or embryo/fetal toxicity.

The BMD_{10} values from the accepted models ranged from 19.9 to 25.2 mg/kg bw per day, and the $BMDL_{10}$ values ranged from 16.8 to 19.8 mg/kg bw per day (Table 16). In order to be prudent, the Committee decided to use the more conservative lower end of this range of values for the evaluation and chose a $BMDL_{10}$ value of 16.8 mg/kg bw per day as the reference point for a margin of exposure (MOE) calculation.

The Committee estimated MOEs assuming a residue level of 0.5 µg/kg, which is a typical limit of quantification for gentian violet residues in foods, and a residue level of 5 µg/kg, which is 10 times the typical limit of quantification, as a hypothetical scenario. Assuming a daily consumption of 300 g of fish contaminated with gentian violet and its metabolites, the estimated theoretical exposures to gentian violet for a 60 kg person were 0.0025 and 0.025 µg/kg bw per day for the two residue levels, respectively. Comparison of these estimated exposures with the $BMDL_{10}$ of 16.8 mg/kg bw per day indicates MOEs of about 6.7×10^6 and 6.7×10^5, respectively. Based on considerations discussed at the sixty-fourth meeting of the Committee for unintended contaminants (Annex 1, reference *176*), these MOEs would be considered to be of low concern for human health.

However, the Committee noted that there were a number of uncertainties associated with the risk assessment, some of which were substantial. The uncertainties relate to two aspects of the data available for risk assessment. Firstly, there were insufficient residue data in food-producing animals or the environment from which to estimate dietary exposure to gentian violet, and hence assumptions had to be made. Secondly, there is very little information on the proportion of gentian violet and its metabolites in the total residue and on the carcinogenicity of the metabolites. For example, there is a published report that one of the possible metabolites of gentian violet, demethylated leucopararosaniline, is carcinogenic in rats, but no information is available on its potency. In addition, there is no information on the carcinogenicity of the major metabolite, leucogentian violet. The structure of gentian violet is similar to that of malachite green, and it is known that leucomalachite green is a more potent carcinogen than malachite green; therefore, it is possible that leucogentian violet is similarly a more potent carcinogen than gentian violet. Gentian violet and leucogentian violet are readily interconvertible in the body, and so it is likely that exposure to gentian violet will also result in exposure to leucogentian violet. Thus, there is inadequate information to determine the overall carcinogenicity of the metabolites of gentian violet (demethylated gentian violet, leucogentian violet and its demethylated metabolites).

5. REFERENCES

Aidoo A, Gao N, Neft RE, Schol HM, Hass BS, Minor TY et al. (1990). Gentian violet in bacterial and mammalian cell systems. Teratogen Carcinogen Mutagen. 10:449–62.

Au W, Hsu TC (1979). Studies on the clastogenic effects of biologic stains and dyes. Environ Mutagen. 1:27–35.

Au W, Pathak S, Collie CJ, Hsu TC (1978). Cytogenetic toxicity of gentian violet and crystal violet on mammalian cells in vitro. Mutat Res. 58:269–76.

Au W, Butler MA, Bloom SE, Matney TS (1979). Further study of the genetic toxicity of gentian violet. Mutat Res. 66:103–12.

Baan R, Straif K, Grosse Y, Secretan B, Ghissassi F, Bouvard V et al. (2008). WHO International Agency for Research on Cancer Monograph Working Group: carcinogenicity of some aromatic amines, organic dyes, and related exposures. Lancet Oncol. 9:322–3.

Bielicky T, Novák MN (1969). Contact-group sensitization to triphenylmethane dyes. Gentian violet, brilliant green, and malachite green. Arch Dermatol. 100:540–3.

Bonin AM, Farquharson GB, Baker RSU (1981). Mutagenicity of arylmethane dyes in *Salmonella*. Mutat Res. 89:21–34.

Dhir SP, Sharma SK, Munjal VP, Gupa A (1982). Keratoconjunctivitis sicca following instillation of gentian violet. Indian J Ophthalmol. 30:21–2.

Diamante C, Bergfeld WF, Belsito DV, Klaassen CD, Marks JG, Shank RC Jr et al. (2009). Final report on the safety assessment of basic violet 1, basic violet 3, and basic violet 4. Int J Toxicol. 28(6 Suppl 2):193S–204S.

Docampo R, Moreno SNJ (1990). The metabolism and mode of action of gentian violet. Drug Metab Rev. 22:161–78.

FAO/WHO (2012). Report of the Twentieth Session of the Codex Committee on Residues of Veterinary Drugs in Foods, San Juan, Puerto Rico, 7–11 May 2012. Rome: Food and Agriculture Organization of the United Nations and World Health Organization, Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission (REP 12/RVDF; http://www.codexalimentarius.org/download/report/778/REP12_RVe.pdf).

Gadelha FR, Moreno SN, De Souza W, Cruz FS, Docampo R (1989). The mitochondrion of *Trypanosoma cruzi* is a target of crystal violet toxicity. Mol Biochem Parasitol. 34:117–26.

Glanville SD, Clark AG (1997). Inhibition of human glutathione *S*-transferases by basic triphenylmethane dyes. Life Sci. 60:1535–44.

Harrelson WG Jr, Mason RP (1982). Microsomal reduction of gentian violet. Evidence for cytochrome P-450 catalyzed free radical formation. Mol Pharmacol. 22:239–42.

Hass BS, Heflich RH, McDonald JJ (1986). Evaluation of the mutagenicity of crystal violet and its metabolites in *Salmonella typhimurium* and *Escherichia coli*. Abstract of the 17th Annual Meeting of the Environmental Mutagen Society, 9–13 April 1986, Baltimore, MD, USA. Abstract No. 95.

Hodge HC, Indra J, Drobeck HP, Duprey LP, Tainter NL (1972). Acute oral toxicity of methylrosaniline chloride. Toxicol Appl Pharmacol. 22:1–5.

Hoffmann ME, Jang J, Moreno SN, Docampo R (1995). Inhibition of protein synthesis and amino acid transport by crystal violet in *Trypanosoma cruzi*. J Eukaryot Microbiol. 42:293–7.

Horsfield P, Logan FA, Newey JA (1976). Oral irritation with gentian violet. Br Med J. 2:529.

Hsu TC, Cherry LM, Pathak S (1982). Induction of chromatid breakage by clastogens in cells of G2 phase. Mutat Res. 93:185–93.

IARC (1993). Occupational exposure of hairdressers and barbers and personal use of hair colorants; some hair dyes, cosmetic colorants, industrial dyestuffs and aromatic amines. IARC [Int Agency Res Cancer] Monogr Eval Carcinog Risk Hum. 57:43–118.

John RW (1968). Necrosis of oral mucosa after local application of crystal violet. Br Med J. 1:157.

Kim SJ, Koh DH, Park JS, Ahn HS, Choi JB, Kim YS (2003). Hemorrhagic cystitis due to intravesical instillation of gentian violet completely recovered with conservative therapy. Yonsei Med J. 44:163–5.

Krishnaja AP, Sharma NK (1995). Heterogeneity in chemical mutagen–induced chromosome damage after G2 phase exposure to bleomycin, ara-C and gentian violet in cultured lymphocytes of β-thalassaemia traits. Mutat Res. 331:143–8.

Lawrence CM, Smith AG (1982). Ampliative medicament allergy: concomitant sensitivity to multiple medicaments including yellow soft paraffin, white soft paraffin, gentian violet and Span 20. Contact Dermatitis. 8:240–5.

Levin DE, Lovely TJ, Klekowski E (1982). Light-enhanced genetic toxicity of crystal violet. Mutat Res. 103:283–8.

Littlefield NA (1984). Chronic toxicity and carcinogenicity studies of gentian violet in mice. Final report. Jefferson (AK): National Center for Toxicological Research, Division of Chemical Toxicology (NCTR Technical Report for Experiment No. 304).

Littlefield NA (1988). Three-generation reproduction and toxicity studies of gentian violet in Fischer 344 rats. Jefferson (AK): National Center for Toxicological Research (NCTR Technical Report for Experiment Nos 305, 354 and 355).

Littlefield NA, Blackwell B-N, Hewitt CC, Gaylor DW (1985). Chronic toxicity and carcinogenicity studies of gentian violet in mice. Fundam Appl Toxicol. 5(5):902–12.

Littlefield NA, Gaylor DW, Blackwell B-N, Allen RR (1989). Chronic toxicity/carcinogenicity studies of gentian violet in Fischer 344 rats: two-generation exposure. Food Chem Toxicol. 27:239–47.

McDonald JJ (1989). Metabolism of gentian violet in Fischer 344 rats and B6C3F1 mice. Jefferson (AK): National Center for Toxicological Research (NCTR Technical Report for Experiment Nos 302 and 303).

McDonald JJ, Cerniglia CE (1984). Biotransformation of gentian violet to leucogentian violet by human, rat, and chicken intestinal microflora. Am Soc Pharmacol Exp Ther. 12:330–6.

McDonald JJ, North CR, Breeden CR, Lai CC, Roth RW (1984a). Synthesis and disposition of ^{14}C-labelled gentian violet in F344 rats and B6C3F1 mice. Food Chem Toxicol. 22:331–6.

McDonald JJ, Breeden CR, North BM, Roth RW (1984b). Species and strain comparison of the metabolism of gentian violet by liver microsomes. J Agric Food Chem. 32:596–600.

Meurer M, Konz B (1977). [Skin necroses following the use of a 2-per-cent Pyoctanin solution.] Hautartzt. 28:94–5 (in German with an English abstract).

Mobacken H, Ahonen J, Zederfeldt B (1974). The effect of cationic triphenylmethane dye (crystal violet) on rabbit granulation tissue. Oxygen consumption and RNA and protein synthesis in tissue slices. Acta Derm Venereol. 54:343–7.

Moreno SNJ, Gadelha FR, Docampo R (1988). Crystal violet as an uncoupler of oxidative phosphorylation in rat liver mitochondria. J Biol Chem. 263:12493–9.

Muller W, Gauthier F (1975). Interactions of heteroaromatic compounds with nucleic acids, A–T-specific non-intercalating DNA ligands. Eur J Biochem. 54:385–94.

National Toxicology Program (1986). Toxicology and carcinogenesis studies of C.I. Basic Red 9 monohydrochloride (pararosaniline) (CAS No. 569-61-9) in F344/N rats and B6C3F1 mice (feed studies). Research Triangle Park (NC): United States Department of Health and Human Services, Public Health Service, National Institutes of Health (NTP Technical Report Series No. 285; http://www.ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr285.pdf) [cited in Docampo & Moreno, 1990].

Nussenzweig V, Sonntag R, Biancalana A, Pedriera de Freitas JL, Amato Neto V, Kloetzel J (1953). Effect of triphenylmethane dyes on *Trypanosoma cruzi* in vitro; use of gentian violet in prevention of Chagas disease by blood transfusion. Hospital (Rio de Janeiro). 44:731–44 [cited in Moreno, Gadelha & Docampo, 1988].

Piatt JP, Bergeson PS (1992). Gentian violet toxicity. Clin Pediatr (Phila). 31:756–7.

Quinby GE (1968). Gentian violet as a cause of epidemic occupational nosebleeds. Arch Environ Health. 16:485–9.

Rollison DE, Helzlsouer KJ, Pinney SM (2006). Personal hair dye use and cancer: a systematic literature review and evaluation of exposure assessment in studies published since 1992. J Toxicol Environ Health. B 9:413–39.

Rosenkranz HS, Carr HS (1971). Possible hazards in use of gentian violet. Br Med J. 3:702–3.

Seppelin [initial unknown] (1949). Unpublished data [cited in Hodge et al., 1972].

Shahin MM, von Borstel RC (1978). Comparisons of mutation induction in reversion systems of *Saccharomyces cerevisiae* and *Salmonella typhimurium*. Mutat Res. 58:1–10.

Slotkowski EL (1957). Formation of mucous membrane lesions secondary to prolonged use of one percent aqueous gentian violet. J Pediatr. 51:652–4.

Slotkowski EL, Redondo D (1966). Mucosal irritation following use of gentian violet. Am J Dis Child. 112:40–2.

Thomas SM, MacPhee DG (1984). Crystal violet: a direct-acting frame shift mutagen whose mutagenicity is enhanced by mammalian metabolism. Mutat Res. 140:165–7.

USFDA (1976). Unpublished data. United States Food and Drug Administration [cited in Littlefield et al., 1989].

Wakelin LPG, Adams A, Hunter C, Waring MJ (1981). Interaction of crystal violet with nucleic acids. Biochemistry. 20:5779–87.

Walsh C, Walsh A (1986). Haemorrhagic cystitis due to gentian violet. Br Med J. 293:732.

Wolkowski-Tyl R, Jones-Price C, Marr MC, Langhoff-Paschke L, Kimmel CA (1982). Teratologic evaluation of gentian violet (CAS No. 548-62-9) in CD rats. Final report. Research Triangle Park (NC): United States Department of Health and Human Services, Public Health Service, National Toxicology Program (NTP Study TER82079).

Wolkowski-Tyl R, Jones-Price C, Reel JR, Marr MC, Langhoff-Paschke L, Kimmel CA (1983). Teratologic evaluation of gentian violet (CAS No. 548-62-9) in New Zealand White rabbits. Final report. Research Triangle Park (NC): United States Department of Health and Human Services, Public Health Service, National Toxicology Program (NTP Study TER82080).

Wright NH, Brady FJ (1940). Studies on oxyuriasis. J Am Med Assoc. 114:861–6 [cited in Hodge et al., 1972].

Appendix C. ToxCast Data for Gentian Violet

OEHHA has organized the ToxCast HTS data for gentian violet, including:

- Table C1: Mapping of ToxCast assays in which gentian violet was active to selected key characteristics of carcinogens
- Table C2: Active ToxCast assays for gentian violet
- Table C3: Active ToxCast assays for pentamethylpararosaniline chloride

Table C1. Mapping of ToxCast assays in which gentian violet was active to selected key characteristics of carcinogens[1]

Assay names	Key characteristic of carcinogens
APR_HepG2_p53Act_24h_up	2. Is Genotoxic
APR_HepG2_p53Act_72h_up	
TOX21_p53_BLA_p1_ratio	
TOX21_p53_BLA_p2_ratio	
TOX21_p53_BLA_p3_ratio	
TOX21_p53_BLA_p4_ratio	
TOX21_p53_BLA_p5_ratio	
ATG_Sp1_CIS_up	4. Induces Epigenetic Alterations
APR_HepG2_OxidativeStress_72h_up	5. Induces Oxidative Stress
APR_HepG2_StressKinase_24h_up	
APR_HepG2_StressKinase_72h_up	
ATG_MRE_CIS_up	
ATG_NRF2_ARE_CIS_up	
TOX21_ARE_BLA_agonist_ratio	
TOX21_HSE_BLA_agonist_ratio	
ATG_ERE_CIS_up	8. Modulates receptor-mediated effects
ATG_HNF4a_TRANS_up	
ATG_PPARg_TRANS_up	
NVS_NR_hPPARg	
NVS_NR_hPXR	
OT_AR_ARSRC1_0480	
TOX21_AR_BLA_Antagonist_ratio	
TOX21_AR_LUC_MDAKB2_Antagonist	
TOX21_AR_LUC_MDAKB2_Antagonist 2	
TOX21_ERa_BLA_Agonist_ratio	
TOX21_ERa_BLA_Antagonist_ratio	
TOX21_ERa_LUC_BG1_Antagonist	
TOX21_FXR_BLA_antagonist_ratio	
TOX21_GR_BLA_Agonist_ratio	
TOX21_GR_BLA_Antagonist_ratio	
TOX21_PPARg_BLA_Agonist_ratio	
TOX21_TR_LUC_GH3_Antagonist	
TOX21_VDR_BLA_antagonist_ratio	

Assay names	Key characteristic of carcinogens
APR_HepG2_ellLoss_72h_dn	10. Alters cell proliferation, cell death or nutrient supply
APR_HepG2_MicrotubuleCSK_24h_dn	
APR_HepG2_MicrotubuleCSK_72h_dn	
APR_HepG2_MitoMass_24h_dn	
APR_HepG2_MitoMass_72h_dn	
APR_HepG2_MitoMembPot_24h_dn	
APR_HepG2_MitoMembPot_72h_dn	
APR_HepG2_MitoticArrest_72h_up	
ATG_AP_1_CIS_up	
ATG_Xbp1_CIS_up	
ATG_XTT_Cytotoxicity_up	
BSK_3C_Proliferation_down	
BSK_3C_SRB_down	
BSK_4H_SRB_down	
BSK_BE3C_SRB_down	
BSK_CASM3C_Proliferation_down	
BSK_CASM3C_SRB_down	
BSK_hDFCGF_Proliferation_down	
BSK_hDFCGF_SRB_down	
BSK_KF3CT_SRB_down	
BSK_LPS_SRB_down	
BSK_SAg_Proliferation_down	
BSK_SAg_SRB_down	
NCCT_HEK293T_CellTiterGLO	
TOX21_AR_BLA_Antagonist_viability	
TOX21_ERa_BLA_Antagonist_viability	
TOX21_FXR_BLA_antagonist_viability	
TOX21_GR_BLA_Antagonist_viability	
TOX21_MMP_ratio_down	
TOX21_MMP_viability	
TOX21_NFkB_BLA_agonist_viability	
TOX21_p53_BLA_p1_viability	
TOX21_p53_BLA_p2_viability	
TOX21_p53_BLA_p3_viability	
TOX21_p53_BLA_p4_viability	

Assay names	Key characteristic of carcinogens
TOX21_p53_BLA_p5_viability	
TOX21_PPARd_BLA_Agonist_viability	
TOX21_VDR_BLA_Agonist_viability	
TOX21_VDR_BLA_antagonist_viability	

[1] Gentian violet tested active in 273 ToxCast assays, and 72 of these 273 assays were mapped to one of the key characteristics of carcinogens.

Table C2. Active ToxCast assays[1] for gentian violet

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
NHEERL_ZF_144hpf_TERATOSCORE_up	null	zebrafish	zebrafish embryo	Developmental defect	0.415
Tanguay_ZF_120hpf_MORT_up	null	zebrafish	dechorionated zebrafish embryo	Developmental defect	0.848
Tanguay_ZF_120hpf_ActivityScore	null	zebrafish	dechorionated zebrafish embryo	Developmental defect	1.56
BSK_SAg_Eselectin_down	SELE	human	umbilical vein endothelium and peripheral blood mononuclear cells	cell adhesion molecules	0.0100
BSK_LPS_Eselectin_down	SELE	human	umbilical vein endothelium and peripheral blood mononuclear cells	cell adhesion molecules	0.0202
BSK_hDFCGF_CollagenIII_down	COL3A1	human	foreskin fibroblast	cell adhesion molecules	0.0417
BSK_4H_Pselectin_down	SELP	human	umbilical vein endothelium	cell adhesion molecules	0.271
BSK_3C_Eselectin_down	SELE	human	umbilical vein endothelium	cell adhesion molecules	0.606
BSK_3C_HLADR_down	HLA-DRA	human	umbilical vein endothelium	cell adhesion molecules	0.692
BSK_CASM3C_SAA_down	SAA1	human	coronary artery smooth muscle cells	cell adhesion molecules	0.901
BSK_BE3C_HLADR_down	HLA-DRA	human	bronchial epithelial cells	cell adhesion molecules	1.51
BSK_hDFCGF_VCAM1_down	VCAM1	human	foreskin fibroblast	cell adhesion molecules	2.03
BSK_LPS_VCAM1_down	VCAM1	human	umbilical vein endothelium and peripheral blood mononuclear cells	cell adhesion molecules	3.07

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
BSK_3C_VCAM1_down	VCAM1	human	umbilical vein endothelium	cell adhesion molecules	4.18
BSK_4H_VCAM1_down	VCAM1	human	umbilical vein endothelium	cell adhesion molecules	4.18
BSK_CASM3C_HLADR_down	HLA-DRA	human	coronary artery smooth muscle cells	cell adhesion molecules	5.15
BSK_3C_ICAM1_down	ICAM1	human	umbilical vein endothelium	cell adhesion molecules	6.05
BSK_CASM3C_VCAM1_down	VCAM1	human	coronary artery smooth muscle cells	cell adhesion molecules	6.08
BSK_KF3CT_ICAM1_down	ICAM1	human	keratinocytes and foreskin fibroblasts	cell adhesion molecules	8.01
BSK_SAg_Proliferation_down	null	human	umbilical vein endothelium and peripheral blood mononuclear cells	cell cycle	0.477
TOX21_AR_BLA_Antagonist_viability	null	human	HEK293T	cell cycle	0.525
APR_HepG2_CellCycleArrest_72h_dn	null	human	HepG2	cell cycle	0.534
NCCT_HEK293T_CellTiterGLO	null	human	HEK293T	cell cycle	0.667
APR_HepG2_CellCycleArrest_24h_dn	null	human	HepG2	cell cycle	0.860
BSK_hDFCGF_Proliferation_down	null	human	foreskin fibroblast	cell cycle	0.947
BSK_hDFCGF_SRB_down	null	human	foreskin fibroblast	cell cycle	1.22
BSK_3C_Proliferation_down	null	human	umbilical vein endothelium	cell cycle	1.64
ATG_XTT_Cytotoxicity_up	null	human	HepG2	cell cycle	10.1
TOX21_p53_BLA_p1_viability	null	human	HCT116	cell cycle	15.4

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
TOX21_p53_BLA_p2_viability	null	human	HCT116	cell cycle	15.7
TOX21_p53_BLA_p5_viability	null	human	HCT116	cell cycle	16.3
TOX21_VDR_BLA_antagonist_viability	null	human	HEK293T	cell cycle	17.4
TOX21_p53_BLA_p3_viability	null	human	HCT116	cell cycle	18.6
TOX21_p53_BLA_p4_viability	null	human	HCT116	cell cycle	18.8
BSK_CASM3C_Proliferation_down	null	human	coronary artery smooth muscle cells	cell cycle	2.27
TOX21_GR_BLA_Antagonist_viability	null	human	HeLa	cell cycle	2.31
ACEA_T47D_80hr_Negative	null	human	T47D	cell cycle	2.72
TOX21_FXR_BLA_antagonist_viability	null	human	HEK293T	cell cycle	21.7
TOX21_NFkB_BLA_agonist_viability	null	human	ME-180	cell cycle	24.6
TOX21_ERa_BLA_Antagonist_viability	null	human	HEK293T	cell cycle	26.1
APR_HepG2_OxidativeStress_24h_up	null	human	HepG2	cell cycle	3.79
BSK_SAg_PBMCCytotoxicity_down	null	human	umbilical vein endothelium and peripheral blood mononuclear cells	cell cycle	4.91
APR_HepG2_StressKinase_72h_up	null	human	HepG2	cell cycle	40.9
APR_HepG2_MitoticArrest_24h_up	null	human	HepG2	cell cycle	5.26
APR_HepG2_OxidativeStress_72h_up	null	human	HepG2	cell cycle	5.31
BSK_KF3CT_SRB_down	null	human	keratinocytes and foreskin fibroblasts	cell cycle	5.36

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
BSK_3C_SRB_down	null	human	umbilical vein endothelium	cell cycle	5.49
APR_HepG2_MitoticArrest_72h_up	null	human	HepG2	cell cycle	5.61
BSK_BE3C_SRB_down	null	human	bronchial epithelial cells	cell cycle	5.71
APR_HepG2_CellLoss_72h_dn	null	human	HepG2	cell cycle	6.05
BSK_CASM3C_SRB_down	null	human	coronary artery smooth muscle cells	cell cycle	6.07
APR_HepG2_StressKinase_24h_up	null	human	HepG2	cell cycle	6.24
BSK_4H_SRB_down	null	human	umbilical vein endothelium	cell cycle	6.80
BSK_SAg_SRB_down	null	human	umbilical vein endothelium and peripheral blood mononuclear cells	cell cycle	7.23
BSK_LPS_SRB_down	null	human	umbilical vein endothelium and peripheral blood mononuclear cells	cell cycle	7.30
TOX21_MMP_viability	null	human	HepG2	cell cycle	70.4
TOX21_PPARd_BLA_Agonist_viability	null	human	HEK293T	cell cycle	9.21
TOX21_VDR_BLA_Agonist_viability	null	human	HEK293T	cell cycle	9.21
APR_HepG2_CellLoss_24h_dn	null	human	HepG2	cell cycle	9.52
APR_HepG2_MitoMass_72h_dn	null	human	HepG2	cell morphology	0.0209
TOX21_MMP_ratio_down	null	human	HepG2	cell morphology	0.0516

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
APR_HepG2_MitoMembPot_72h_dn	null	human	HepG2	cell morphology	0.438
BSK_3C_Vis_down	null	human	umbilical vein endothelium	cell morphology	12.3
APR_HepG2_MicrotubuleCSK_24h_dn	null	human	HepG2	cell morphology	17.1
APR_HepG2_MitoMass_24h_dn	null	human	HepG2	cell morphology	4.19
APR_HepG2_MitoMembPot_24h_dn	null	human	HepG2	cell morphology	80.1
APR_HepG2_MicrotubuleCSK_72h_dn	null	human	HepG2	cell morphology	9.94
TOX21_Aromatase_Inhibition	CYP19A1	human	MCF-7	cyp	0.495
NVS_ADME_hCYP1A1	CYP1A1	human	NA	cyp	1.82
TOX21_VDR_BLA_antagonist_ratio	CYP24A1	human	HEK293T	cyp	24.7
NVS_ADME_hCYP2D6	CYP2D6	human	NA	cyp	3.60
NVS_ADME_hCYP2C19	CYP2C19	human	NA	cyp	3.86
NVS_ADME_hCYP2B6	CYP2B6	human	NA	cyp	5.08
NVS_ADME_hCYP3A4	CYP3A4	human	NA	cyp	6.99
NVS_ADME_hCYP1A2	CYP1A2	human	NA	cyp	7.76
NVS_ADME_hCYP2C9	CYP2C9	human	NA	cyp	8.51
BSK_LPS_IL1a_down	IL1A	human	umbilical vein endothelium and peripheral blood mononuclear cells	cytokine	0.0100
BSK_SAg_IL8_down	CXCL8	human	umbilical vein endothelium and peripheral blood mononuclear cells	cytokine	0.0100
BSK_SAg_MCP1_down	CCL2	human	umbilical vein endothelium and peripheral blood	cytokine	0.0226

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
			mononuclear cells		
BSK_LPS_CD40_down	CD40	human	umbilical vein endothelium and peripheral blood mononuclear cells	cytokine	0.0395
BSK_LPS_MCSF_down	CSF1	human	umbilical vein endothelium and peripheral blood mononuclear cells	cytokine	0.179
BSK_BE3C_PAI1_down	SERPINE1	human	bronchial epithelial cells	cytokine	0.300
BSK_SAg_CD40_down	CD40	human	umbilical vein endothelium and peripheral blood mononuclear cells	cytokine	0.312
BSK_LPS_TissueFactor_down	F3	human	umbilical vein endothelium and peripheral blood mononuclear cells	cytokine	0.343
BSK_LPS_MCP1_down	CCL2	human	umbilical vein endothelium and peripheral blood mononuclear cells	cytokine	0.438
BSK_4H_MCP1_down	CCL2	human	umbilical vein endothelium	cytokine	0.651
BSK_3C_MCP1_down	CCL2	human	umbilical vein endothelium	cytokine	0.652
BSK_CASM3C_TissueFactor_down	F3	human	coronary artery smooth muscle cells	cytokine	0.694
BSK_SAg_CD38_down	CD38	human	umbilical vein endothelium and peripheral blood	cytokine	0.713

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
			mononuclear cells		
BSK_4H_Eotaxin3_down	CCL26	human	umbilical vein endothelium	cytokine	0.811
BSK_SAg_CD69_down	CD69	human	umbilical vein endothelium and peripheral blood mononuclear cells	cytokine	0.821
BSK_3C_uPAR_down	PLAUR	human	umbilical vein endothelium	cytokine	0.840
BSK_LPS_IL8_down	CXCL8	human	umbilical vein endothelium and peripheral blood mononuclear cells	cytokine	0.961
BSK_4H_uPAR_down	PLAUR	human	umbilical vein endothelium	cytokine	0.980
BSK_LPS_TNFa_down	TNF	human	umbilical vein endothelium and peripheral blood mononuclear cells	cytokine	0.998
BSK_hDFCGF_PAI1_down	SERPINE1	human	foreskin fibroblast	cytokine	1.05
BSK_CASM3C_uPAR_down	PLAUR	human	coronary artery smooth muscle cells	cytokine	1.06
BSK_SAg_MIG_down	CXCL9	human	umbilical vein endothelium and peripheral blood mononuclear cells	cytokine	1.08
BSK_hDFCGF_MCSF_down	CSF1	human	foreskin fibroblast	cytokine	1.21

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
BSK_KF3CT_IL1a_down	IL1A	human	keratinocytes and foreskin fibroblasts	cytokine	2.12
BSK_3C_TissueFactor_down	F3	human	umbilical vein endothelium	cytokine	2.20
BSK_CASM3C_MCP1_down	CCL2	human	coronary artery smooth muscle cells	cytokine	2.55
BSK_BE3C_IL1a_down	IL1A	human	bronchial epithelial cells	cytokine	2.81
BSK_3C_IL8_down	CXCL8	human	umbilical vein endothelium	cytokine	3.19
BSK_hDFCGF_IP10_down	CXCL10	human	foreskin fibroblast	cytokine	3.49
BSK_KF3CT_MCP1_down	CCL2	human	keratinocytes and foreskin fibroblasts	cytokine	3.53
BSK_CASM3C_IL8_down	CXCL8	human	coronary artery smooth muscle cells	cytokine	4.26
BSK_hDFCGF_MIG_down	CXCL9	human	foreskin fibroblast	cytokine	4.30
BSK_BE3C_uPAR_down	PLAUR	human	bronchial epithelial cells	cytokine	4.34
BSK_hDFCGF_IL8_down	CXCL8	human	foreskin fibroblast	cytokine	4.77
BSK_CASM3C_MCSF_down	CSF1	human	coronary artery smooth muscle cells	cytokine	4.98
BSK_CASM3C_IL6_down	IL6	human	coronary artery smooth muscle cells	cytokine	5.15
BSK_BE3C_MIG_down	CXCL9	human	bronchial epithelial cells	cytokine	5.80
BSK_KF3CT_IP10_down	CXCL10	human	keratinocytes and foreskin fibroblasts	cytokine	5.90
BSK_BE3C_IP10_down	CXCL10	human	bronchial epithelial cells	cytokine	6.40

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
BSK_CASM3C_MIG_down	CXCL9	human	coronary artery smooth muscle cells	cytokine	7.29
BSK_3C_MIG_down	CXCL9	human	umbilical vein endothelium	cytokine	7.30
ATG_HIF1a_CIS_dn	null	human	HepG2	DNA binding	0.000841
ATG_p53_CIS_dn	null	human	HepG2	DNA binding	0.904
ATG_E2F_CIS_dn	null	human	HepG2	DNA binding	1.05
ATG_Sp1_CIS_up	SP1	human	HepG2	DNA binding	1.45
ATG_HNF6_CIS_up	ONECUT1	human	HepG2	DNA binding	1.61
ATG_ISRE_CIS_dn	null	human	HepG2	DNA binding	1.71
ATG_AP_1_CIS_up	JUN, FOS	human	HepG2	DNA binding	1.76
ATG_NRF2_ARE_CIS_up	NFE2L2	human	HepG2	DNA binding	1.76
ATG_EGR_CIS_up	EGR1	human	HepG2	DNA binding	1.81
TOX21_ARE_BLA_agonist_ratio	NFE2L2	human	HepG2	DNA binding	1.90
TOX21_p53_BLA_p1_ratio	TP53	human	HCT116	DNA binding	13.9
ATG_Xbp1_CIS_up	XBP1	human	HepG2	DNA binding	2.01
ATG_TCF_b_cat_CIS_dn	null	human	HepG2	DNA binding	2.35
ATG_AP_2_CIS_up	TFAP2A, TFAP2B, TFAP2D	human	HepG2	DNA binding	2.39
ATG_E_Box_CIS_dn	null	human	HepG2	DNA binding	2.68
TOX21_p53_BLA_p5_ratio	TP53	human	HCT116	DNA binding	26.5
APR_HepG2_p53Act_72h_up	TP53	human	HepG2	DNA binding	29.2
TOX21_p53_BLA_p4_ratio	TP53	human	HCT116	DNA binding	29.2
APR_HepG2_p53Act_24h_up	TP53	human	HepG2	DNA binding	3.01
ATG_MRE_CIS_up	MTF1	human	HepG2	DNA binding	3.06
ATG_C_EBP_CIS_dn	null	human	HepG2	DNA binding	3.66

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
TOX21_HSE_BLA_agonist_ratio	HSF1	human	HeLa	DNA binding	4.27
ATG_HSE_CIS_up	HSF1	human	HepG2	DNA binding	5.09
TOX21_p53_BLA_p2_ratio	TP53	human	HCT116	DNA binding	6.08
TOX21_p53_BLA_p3_ratio	TP53	human	HCT116	DNA binding	7.13
NVS_ENZ_hES	BCHE	human	NA	esterase	1.73
NVS_ENZ_rAChE	Ache	rat	NA	esterase	23.4
NVS_ENZ_hAChE	ACHE	human	NA	esterase	7.02
BSK_LPS_PGE2_down	PTGER2	human	umbilical vein endothelium and peripheral blood mononuclear cells	GPCR	0.792
NVS_GPCR_gH2	Hrh2	guinea pig	NA	GPCR	0.834
NVS_GPCR_hM5	CHRM5	human	NA	GPCR	0.871
NVS_GPCR_hM4	CHRM4	human	NA	GPCR	0.885
NVS_GPCR_hM2	CHRM2	human	NA	GPCR	0.961
NVS_GPCR_hDRD4.4	DRD4	human	NA	GPCR	1.14
NVS_GPCR_rAdra2_NonSelective	Adra2a	rat	NA	GPCR	1.20
NVS_GPCR_hETA	EDNRA	human	NA	GPCR	1.80
NVS_GPCR_hAdra2C	ADRA2C	human	NA	GPCR	1.91
NVS_GPCR_hM1	CHRM1	human	NA	GPCR	1.96
NVS_GPCR_g5HT4	Htr4	guinea pig	NA	GPCR	10.2
NVS_GPCR_rOpiate_NonSelectiveNa	Oprm1	rat	NA	GPCR	10.4
NVS_GPCR_rmAdra2B	Adra2b	rat	NA	GPCR	11.8
NVS_GPCR_rNTS	Ntsr1	rat	NA	GPCR	12.1
NVS_GPCR_hAdoRA1	ADORA1	human	NA	GPCR	12.4
NVS_GPCR_hLTB4_BLT1	LTB4R	human	NA	GPCR	12.7

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
NVS_GPCR_mCCKAPeripheral	Cckar	mouse	NA	GPCR	14.9
NVS_GPCR_bNPY_NonSelective	NPY	bovine	NA	GPCR	15.1
NVS_GPCR_gBK2	Bdkrb2	guinea pig	NA	GPCR	16.1
NVS_GPCR_rNK3	Tacr3	rat	NA	GPCR	16.1
NVS_GPCR_bAdoR_NonSelective	ADORA1	bovine	NA	GPCR	16.3
NVS_GPCR_mCKKBCentral	Cckbr	mouse	NA	GPCR	17.1
NVS_GPCR_h5HT2A	HTR2A	human	NA	GPCR	17.4
NVS_GPCR_h5HT6	HTR6	human	NA	GPCR	17.8
NVS_GPCR_rNK1	Tacr1	rat	NA	GPCR	18.2
NVS_GPCR_hNPY1	NPY1R	human	NA	GPCR	18.5
NVS_GPCR_p5HT2C	HTR2C	pig	NA	GPCR	19.1
NVS_GPCR_rAdrb_NonSelective	Adrb1	rat	NA	GPCR	19.2
NVS_GPCR_hAdrb2	ADRB2	human	NA	GPCR	19.3
NVS_GPCR_hAdrb3	ADRB3	human	NA	GPCR	19.5
NVS_GPCR_hNPY2	NPY2R	human	NA	GPCR	19.7
NVS_GPCR_hDRD2s	DRD2	human	NA	GPCR	2.10
NVS_GPCR_hDRD1	DRD1	human	NA	GPCR	2.18
NVS_GPCR_h5HT5A	HTR5A	human	NA	GPCR	2.22
NVS_GPCR_rOpiate_NonSelective	Oprm1	rat	NA	GPCR	2.39
NVS_GPCR_hAdra2A	ADRA2A	human	NA	GPCR	2.52
NVS_GPCR_hOpiate_mu	OPRM1	human	NA	GPCR	2.78
NVS_GPCR_bDR_NonSelective	DRD1	bovine	NA	GPCR	24.0
NVS_GPCR_rAdra1A	Adra1a	rat	NA	GPCR	25.2
NVS_GPCR_rOXT	Oxtr	rat	NA	GPCR	25.6

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
NVS_GPCR_hORL1	OPRL1	human	NA	GPCR	26.9
NVS_GPCR_r5HT_NonSelective	Htr1a	rat	NA	GPCR	27.5
NVS_GPCR_rH3	Hrh3	rat	NA	GPCR	28.2
NVS_GPCR_hNK2	TACR2	human	NA	GPCR	4.07
NVS_GPCR_h5HT7	HTR7	human	NA	GPCR	4.41
BSK_3C_Thrombomodulin_down	THBD	human	umbilical vein endothelium	GPCR	4.71
NVS_GPCR_gOpiateK	Oprk1	guinea pig	NA	GPCR	4.98
NVS_GPCR_gMPeripheral_NonSelective	Chrm3	guinea pig	NA	GPCR	5.41
NVS_GPCR_rAdra1_NonSelective	Adra1a	rat	NA	GPCR	5.43
NVS_GPCR_rAdra1B	Adra1b	rat	NA	GPCR	5.56
NVS_GPCR_hM3	CHRM3	human	NA	GPCR	6.43
NVS_GPCR_hH1	HRH1	human	NA	GPCR	7.26
BSK_CASM3C_Thrombomodulin_down	THBD	human	coronary artery smooth muscle cells	GPCR	8.29
NVS_GPCR_hAdrb1	ADRB1	human	NA	GPCR	9.07
NVS_GPCR_hOpiate_D1	OPRD1	human	NA	GPCR	9.22
BSK_BE3C_TGFb1_down	TGFB1	human	bronchial epithelial cells	growth factor	0.612
BSK_KF3CT_TGFb1_down	TGFB1	human	keratinocytes and foreskin fibroblasts	growth factor	0.829
NVS_IC_rNaCh_site2	Scn1a	rat	NA	ion channel	0.255
NVS_LGIC_bGABAR_Agonist	GABRA1	bovine	NA	ion channel	14.5
NVS_LGIC_rNNR_BungSens	Chrna7	rat	NA	ion channel	19.5
NVS_LGIC_h5HT3	HTR3A	human	NA	ion channel	20.1
NVS_LGIC_rAMPA	Gria1	rat	NA	ion channel	21.9

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
NVS_IC_rKCaCh	Kcnn1	rat	NA	ion channel	22.3
NVS_IC_hKhERGCh	KCNH2	human	NA	ion channel	5.75
NVS_IC_rCaBTZCHL	Cacna1a	rat	NA	ion channel	5.87
NVS_IC_rCaDHPRCh_L	Cacna1a	rat	NA	ion channel	6.86
BSK_4H_VEGFRII_down	KDR	human	umbilical vein endothelium	kinase	0.0182
NVS_ENZ_hVEGFR2	KDR	human	NA	kinase	0.562
BSK_hDFCGF_EGFR_down	EGFR	human	foreskin fibroblast	kinase	1.55
NVS_OR_gSIGMA_NonSelective	Sigmar1	guinea pig	NA	misc protein	15.4
BSK_CASM3C_LDLR_down	LDLR	human	coronary artery smooth muscle cells	misc protein	7.17
TOX21_AR_BLA_Antagonist_ratio	AR	human	HEK293T	nuclear receptor	0.269
TOX21_ERa_BLA_Antagonist_ratio	ESR1	human	HEK293T	nuclear receptor	0.303
TOX21_TR_LUC_GH3_Antagonist	THRB, THRA	rat	GH3	nuclear receptor	0.351
NVS_NR_hPPARg	PPARG	human	NA	nuclear receptor	0.816
ATG_ERE_CIS_up	ESR1	human	HepG2	nuclear receptor	0.864
TOX21_AR_LUC_MDAKB2_Antagonist	AR	human	MDA-kb2	nuclear receptor	1.30
ATG_PXRE_CIS_dn	null	human	HepG2	nuclear receptor	1.82
ATG_PPRE_CIS_dn	null	human	HepG2	nuclear receptor	1.85
TOX21_PPARg_BLA_Agonist_ratio	PPARG	human	HEK293T	nuclear receptor	15.7
ATG_HNF4a_TRANS_up	HNF4A	human	HepG2	nuclear receptor	16.4
NVS_NR_hPPARa	PPARA	human	NA	nuclear receptor	17.1
TOX21_FXR_BLA_antagonist_ratio	NR1H4	human	HEK293T	nuclear receptor	17.3

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
NVS_NR_cAR	AR	chimpanzee	NA	nuclear receptor	18.8
ATG_DR4_LXR_CIS_dn	null	human	HepG2	nuclear receptor	2.07
ATG_DR5_CIS_dn	null	human	HepG2	nuclear receptor	2.08
NVS_NR_hGR	NR3C1	human	NA	nuclear receptor	2.10
ATG_IR1_CIS_dn	null	human	HepG2	nuclear receptor	2.29
TOX21_GR_BLA_Antagonist_ratio	NR3C1	human	HeLa	nuclear receptor	2.81
TOX21_ERa_LUC_BG1_Antagonist	ESR1	human	BG1	nuclear receptor	2.99
TOX21_GR_BLA_Agonist_ratio	NR3C1	human	HeLa	nuclear receptor	34.8
ATG_PBREM_CIS_dn	null	human	HepG2	nuclear receptor	4.33
NVS_NR_bPR	PGR	bovine	NA	nuclear receptor	4.35
ATG_RARa_TRANS_dn	null	human	HepG2	nuclear receptor	4.39
NVS_NR_rAR	Ar	rat	NA	nuclear receptor	5.18
ATG_PPARa_TRANS_dn	null	human	HepG2	nuclear receptor	5.63
NVS_NR_hPXR	NR1I2	human	NA	nuclear receptor	5.72
ATG_RORb_TRANS_dn	null	human	HepG2	nuclear receptor	6.09
ATG_CAR_TRANS_dn	null	human	HepG2	nuclear receptor	6.37
OT_AR_ARSRC1_0480	AR	human	HEK293T	nuclear receptor	6.59
TOX21_ERa_BLA_Agonist_ratio	ESR1	human	HEK293T	nuclear receptor	8.40
ATG_FXR_TRANS_dn	null	human	HepG2	nuclear receptor	9.76
ATG_PPARg_TRANS_up	PPARG	human	HepG2	nuclear receptor	9.76
NCCT_TPO_AUR_dn	Tpo	rat	NA	oxidoreductase	15.0
NCCT_QuantiLum_inhib_dn	null	NA	NA	oxidoreductase	2.60
NVS_ENZ_rMAOBP	Maob	rat	NA	oxidoreductase	28.8
NVS_ENZ_rMAOAC	Maoa	rat	NA	oxidoreductase	28.9
NCCT_QuantiLum_inhib_2_dn	null	NA	NA	oxidoreductase	5.12

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
BSK_BE3C_MMP1_down	MMP1	human	bronchial epithelial cells	protease	0.648
BSK_BE3C_tPA_down	PLAT	human	bronchial epithelial cells	protease	0.747
BSK_KF3CT_MMP9_down	MMP9	human	keratinocytes and foreskin fibroblasts	protease	1.37
BSK_KF3CT_uPA_down	PLAU	human	keratinocytes and foreskin fibroblasts	protease	1.98
NVS_ENZ_hBACE	BACE1	human	NA	protease	13.9
NVS_ENZ_hMMP9	MMP9	human	NA	protease	16.1
BSK_BE3C_uPA_down	PLAU	human	bronchial epithelial cells	protease	2.20
NVS_ENZ_hCASP2	CASP2	human	NA	protease	20.8
BSK_hDFCGF_MMP1_down	MMP1	human	foreskin fibroblast	protease	3.19
BSK_KF3CT_TIMP2_down	TIMP2	human	keratinocytes and foreskin fibroblasts	protease inhibitor	0.953
BSK_hDFCGF_TIMP1_down	TIMP1	human	foreskin fibroblast	protease inhibitor	1.19
NVS_MP_hPBR	TSPO	human	NA	transporter	1.48
NVS_TR_rVMAT2	Slc18a2	rat	NA	transporter	2.03
NVS_TR_hDAT	SLC6A3	human	NA	transporter	5.22
NVS_TR_hNET	SLC6A2	human	NA	transporter	5.43
NVS_TR_hSERT	SLC6A4	human	NA	transporter	6.77
NVS_TR_gDAT	Slc6a3	guinea pig	NA	transporter	8.32
NVS_MP_rPBR	Tspo	rat	NA	transporter	8.38
NVS_TR_rSERT	Slc6a4	rat	NA	transporter	9.25
NVS_TR_hAdoT	SLC29A1	human	NA	transporter	9.61

[1] Alphabetically ordered by “intended target families”. This table does not include results classified by US EPA as ‘background measurement’ assays (i.e., artifact fluorescence, baseline controls, and internal markers). AC50: the concentration that induces a half-maximal assay response.

Table C3. Active ToxCast assays[1] for pentamethylpararosaniline chloride[2]

Assay Name	Gene Symbol	Organism	Cells/ Cell Lines	Intended Target Family	AC50 (µM)
TOX21_ERa_BLA_Antagonist_viability	AHR	human	HEK293T	cell cycle	12.2
TOX21_p53_BLA_p4_viability	AR	human	HCT116	cell cycle	17.9
TOX21_AR_BLA_Antagonist_viability	null	human	HEK293T	cell cycle	2.19
TOX21_p53_BLA_p2_viability	AR	human	HCT116	cell cycle	23.3
TOX21_p53_BLA_p3_viability	CYP19A1	human	HCT116	cell cycle	29.5
TOX21_GR_BLA_Antagonist_viability	ESR1	human	HeLa	cell cycle	9.96
TOX21_Aromatase_Inhibition	ESR1	human	MCF-7	cyp	1.42
TOX21_p53_BLA_p3_ratio	null	human	HCT116	DNA binding	12.6
TOX21_p53_BLA_p1_ratio	ESR1	human	HCT116	DNA binding	28.6
TOX21_p53_BLA_p4_ratio	NR3C1	human	HCT116	DNA binding	32.3
TOX21_AhR_LUC_Agonist	null	human	HepG2	DNA binding	5.35
TOX21_p53_BLA_p2_ratio	TP53	human	HCT116	DNA binding	6.17
TOX21_p53_BLA_p5_ratio	TP53	human	HCT116	DNA binding	8.51
TOX21_AR_BLA_Antagonist_ratio	null	human	HEK293T	nuclear receptor	0.294
TOX21_ERa_BLA_Antagonist_ratio	TP53	human	HEK293T	nuclear receptor	1.81
TOX21_TR_LUC_GH3_Antagonist	null	rat	GH3	nuclear receptor	2.38
TOX21_ERa_BLA_Agonist_ratio	TP53	human	HEK293T	nuclear receptor	27.7
TOX21_AR_LUC_MDAKB2_Antagonist	null	human	MDA-kb2	nuclear receptor	3.54
TOX21_ERa_LUC_BG1_Antagonist	TP53	human	BG1	nuclear receptor	7.02
TOX21_GR_BLA_Antagonist_ratio	PPARG	human	HeLa	nuclear receptor	7.67
TOX21_PPARg_BLA_Agonist_ratio	THRB, THRA	human	HEK293T	nuclear receptor	8.32

[1] Alphabetically ordered by “intended target families”. This table does not include results classified by US EPA as ‘background measurement’ assays (i.e., artifact fluorescence, baseline controls, and internal markers).

[2] Purity of pentamethylpararosaniline chloride used in these assays was reported to be < 14%.

AC50: the concentration that induces a half-maximal assay response.