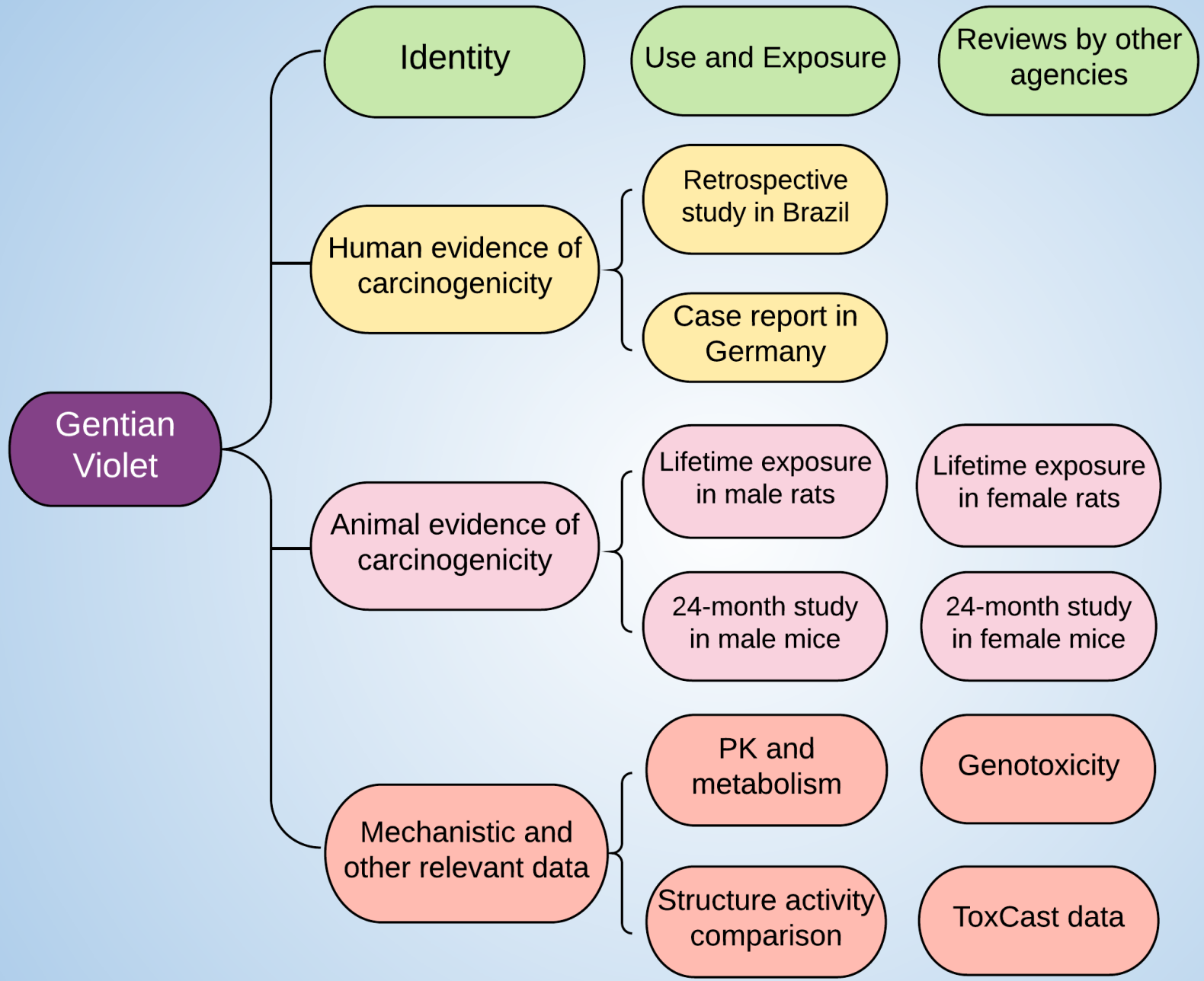
Evidence on the Carcinogenicity of Gentian Violet

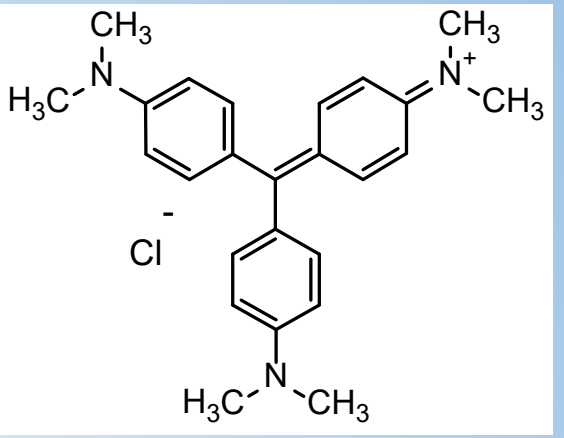
Carcinogen Identification Committee Meeting
November 1st, 2018

Meng Sun, Karin Ricker, Gwendolyn Osborne,
Elizabeth Marder, and Rose Schmitz

Cancer Toxicology and Epidemiology Section
Reproductive and Cancer Hazard Assessment Branch
CalEPA Office of Environmental Health Hazard Assessment


Gentian Violet
Identity
Use and Exposure
Reviews by other agencies
Human evidence of carcinogenicity
Retrospective study in Brazil
Case report in Germany
Animal evidence of carcinogenicity
Lifetime exposure in male rats
Lifetime exposure in female rats
24-month study in male mice
24-month study in female mice
Mechanistic and other relevant data
PK and metabolism
Genotoxicity
Structure activity comparison
ToxCast data

Use and Exposure



- Histological and biological stain
  - Gram stain
  - Nuclear stain
- Commercial dye for paper, textiles, elastic fibers, inks, and toners
- Varied uses based on antimicrobial properties
  - Anti-bacterial foams & topical solutions for wound healing & first aid
    - Other uses of topical solutions:
      - ❖ Treatment of infant oral thrush & thrush of the nipple
      - ❖ Consumers' adaptation for cosmetic use
  - Non-permitted uses for aqua-cultured seafood outside US
    - Potential consumption of adulterated seafood

Statements by other agencies

- Food and Drug Administration (1980; 2008)
  - "Gentian violet is a suspected carcinogen, a probable mutagen, and a potent clastogen"
- National Toxicology Program (2005)
  - Referred to gentian violet as a "carcinogenic dye" in its technical report on the carcinogenicity of two structurally related compounds -- malachite green chloride and leucomalachite green
- Joint FAO/WHO Expert Committee on Food Additives (2014)
  - "it is inappropriate to set an ADI [acceptable daily intake] for gentian violet because it is genotoxic and carcinogenic"
- Australian Pesticides and Veterinary Medicines Authority (2014)
  - "crystal (gentian) violet demonstrated carcinogenic/tumorigenic effects in mice in life-span studies" AND "(gentian) violet is a mutagen and clastogen"
    - Cancelled registrations and approvals of products containing crystal (gentian) violet

Human Data

- Hospital-based retrospective study (De Sousa et al. 1989)
  - 4,765 patients interviewed. Of 37 patients who recalled receiving gentian violet treated blood, 26 had benign or malignant neoplastic lesions.
  - Limitations
    - Lack of information on site and type of cancers observed, and on comparison group
    - Selection bias (patients from a hospital affiliated with “combating cancer”)
    - Confounding factors (higher iron levels, immunosuppression in blood transfusion recipients)

Carcinogenicity Studies in Animals
Gentian Violet (Purity: 99%)

Species	Strain	Sex	Route & Exposure Length	Reference
Rat	F344	Male	*In utero*, during lactation, and via feed post-weaning for up to 24 months	Littlefield et al. 1989 NCTR 1988
		Female		
Mouse	B6C3F1	Male	Via feed from 4-5 weeks of age for up to 24 months	Littlefield et al. 1985 NCTR 1983
		Female		

Tumor incidence in male F344 rats (F_1)

(Littlefield et al. 1989; NCTR 1988)

***In utero*, during lactation, and via feed post-weaning for up to 24 months**

Tumor type	Timing of assessment	Concentration in feed (ppm)				Trend test p-value
		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	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**	p<0.01
Testis and epididymis mesothelioma	18 months	0	0	13%	13%	NA
	Up to 24 months	3%	2%	6%	9%	NA

NS, not significant; NA, not applicable
* p<0.05, ** p<0.01

Tumor incidence in female F344 rats (F_1)

(Littlefield et al. 1989; NCTR 1988)

***In utero*, during lactation, and via feed post-weaning for up to 24 months**

Tumor type	Timing of assessment	Concentration in feed (ppm)				Trend test p-value
		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)	Up to 24 months	12%	6%	18%	33%	NA

NS, not significant; NA, not applicable
* p<0.05, ** p<0.01, *** p<0.001

Tumor incidence in male B6C3F1 mice

(Littlefield et al. 1985; NCTR 1983)

Via feed, up to 24 months

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

NS, not significant

* p<0.05, ** p<0.01, *** p<0.001

Tumor incidence in female B6C3F1 mice

(Littlefield et al. 1985; NCTR 1983) Via feed, up to 24 months

Tumor type	Timing of assessment	Concentration in feed (ppm)				Trend test p-value
		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

NS, not significant

* p<0.05, ** p<0.01, *** p<0.001

Tumor incidence in female B6C3F1 mice (continued)

(Littlefield et al. 1985; NCTR 1983) Via feed, up to 24 months

Tumor type	Timing of assessment	Concentration in feed (ppm)				Trend test p-value
		0	100	300	600	
Reticulum cell sarcoma (type A), 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), 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), 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), 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

NS, not significant

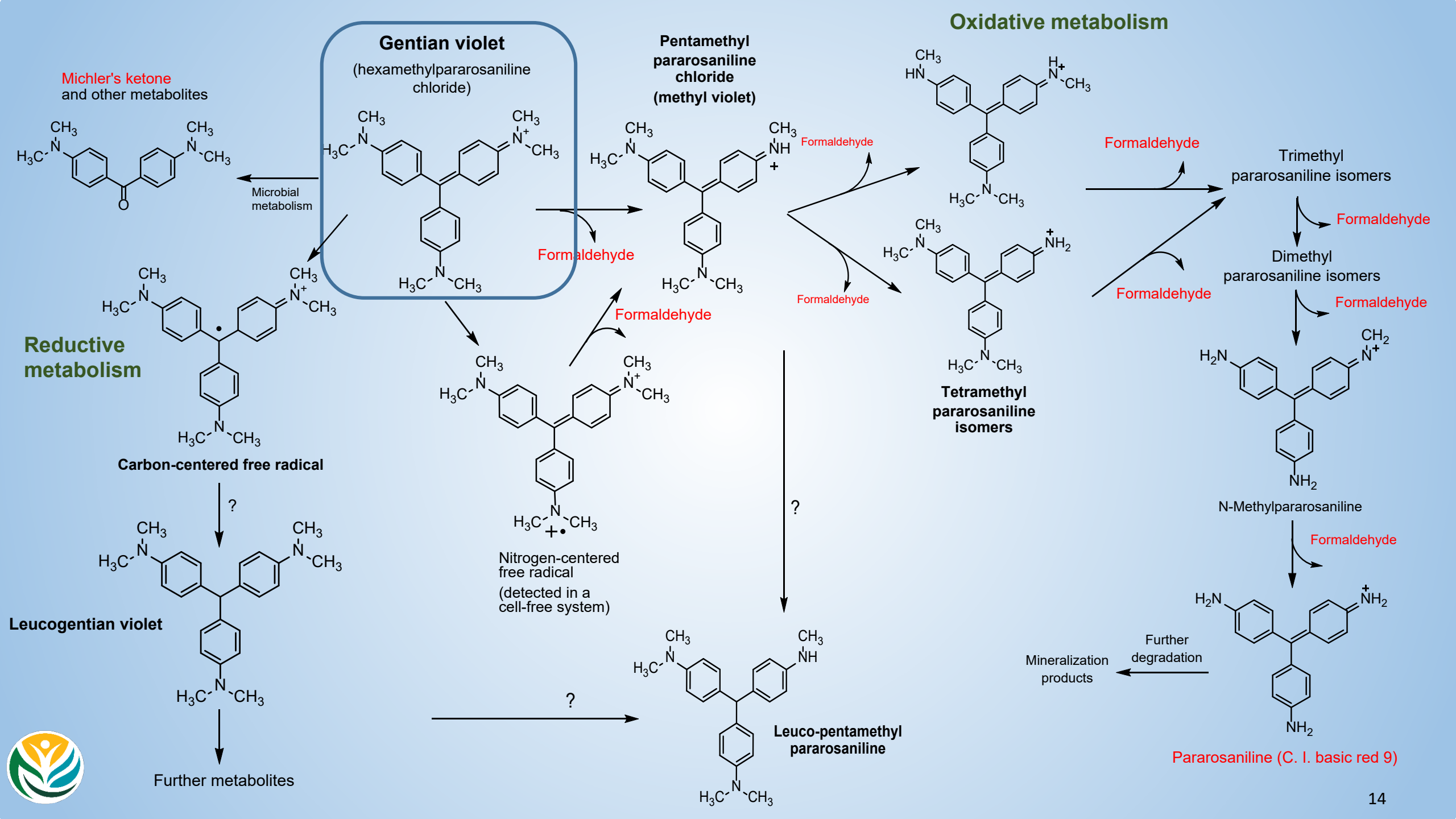
* p<0.05, ** p<0.01, *** p<0.001

Pharmacokinetics & metabolism

- Human studies
  - *In vivo* - none
  - *In vitro* - intestinal microflora
- Animal studies
  - *In vivo* - rats (oral), mice (oral), chicken (oral)
  - *In vitro* - liver microsomes from rats (3 strains), mice (4 strains), hamster, guinea pig, chicken; intestinal microflora (rat, chicken)
- Other
  - Microbial studies
  - Cell free systems (light, horseradish peroxidase)

Absorption, distribution & excretion

- Rapid but incomplete absorption by oral route
  - Less than 10 % of dose is absorbed within 2 hours (rats)
- Distribution
  - Rapid distribution of parent compound and metabolites
    - Highest levels in liver, kidney & adipose tissue
- Excretion (rodents)
  - Biliary excretion 5.7 - 6.4 %
  - Fecal excretion 63.8 - 72.9 %
  - Urinary excretion 2.2 - 8.1 %


Gentian violet
(hexamethylpararosaniline chloride)
Michler's ketone
and other metabolites
Microbial metabolism
Pentamethyl pararosaniline chloride
(methyl violet)
Formaldehyde
Oxidative metabolism
Tetramethyl pararosaniline isomers
Trimethyl pararosaniline isomers
Dimethyl pararosaniline isomers
N-Methylpararosaniline
Pararosaniline (C. I. basic red 9)
Reductive metabolism
Carbon-centered free radical
Nitrogen-centered free radical
(detected in a cell-free system)
Leucogentian violet
Further metabolites
Leuco-pentamethyl pararosaniline
Further degradation
Mineralization products
?

Genotoxicity Studies of Gentian Violet

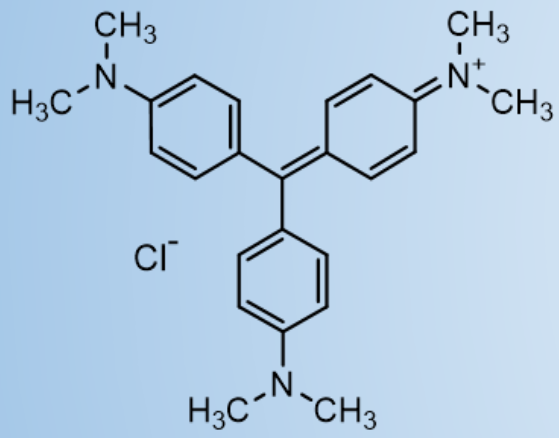
- Mutations
  - in *Salmonella typhimurium* TA97, TA98, TA100, TA104, and TA1535
  - in *E. coli*
- DNA damage
  - in *B. subtilis* and *E. coli*
  - in mouse lymphocytes
- Clastogenicity
  - CAs in CHO, human lymphocytes and HeLa cells, and other mammalian cells
  - Chromosome breakage in CHO and human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

Genotoxic Metabolites of Gentian Violet

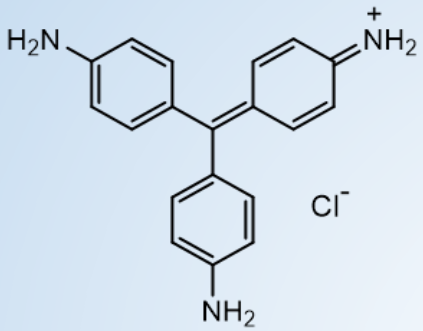
- Pentamethylpararosaniline chloride
  - Is mutagenic in bacteria and bacteriophage
  - Binds to calf thymus DNA
- Leucogentian violet and leuco-pentamethylpararosaniline
  - Are mutagenic in *Salmonella*
- N,N,N',N'- and N,N,N',N''-tetramethylpararosaniline
  - Are mutagenic in *Salmonella* and *E. coli*
- Formaldehyde, C.I. Basic Red 9, and Michler's ketone
  - The latter two are microbial metabolites of gentian violet, and may be produced by intestinal microflora
  - All three carcinogens are genotoxic with a variety of endpoints

Gentian Violet and Structurally-Related Chemicals

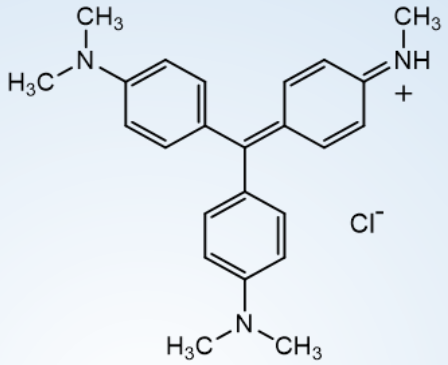
Gentian Violet



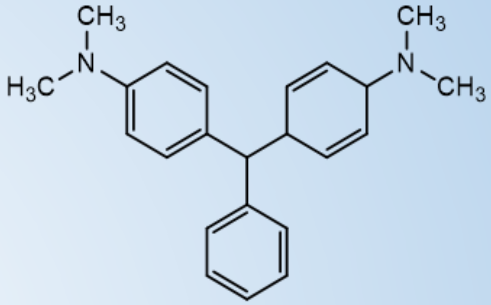
C.I. Basic Red 9



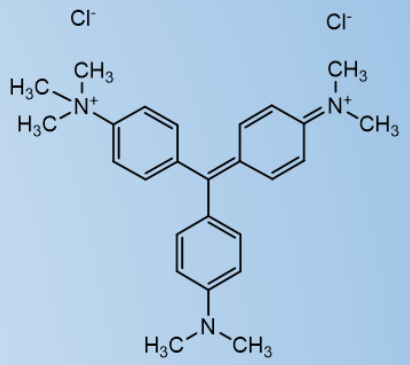
Pentamethylpararosaniline chloride



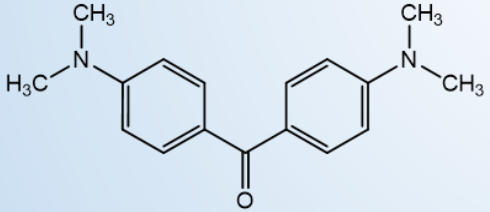
Leucomalachite Green



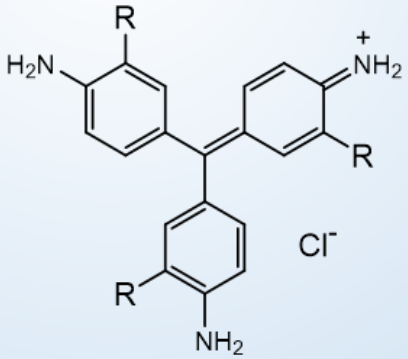
Methyl Green



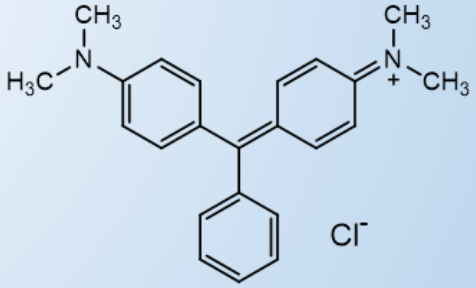
Michler's Ketone



Magenta



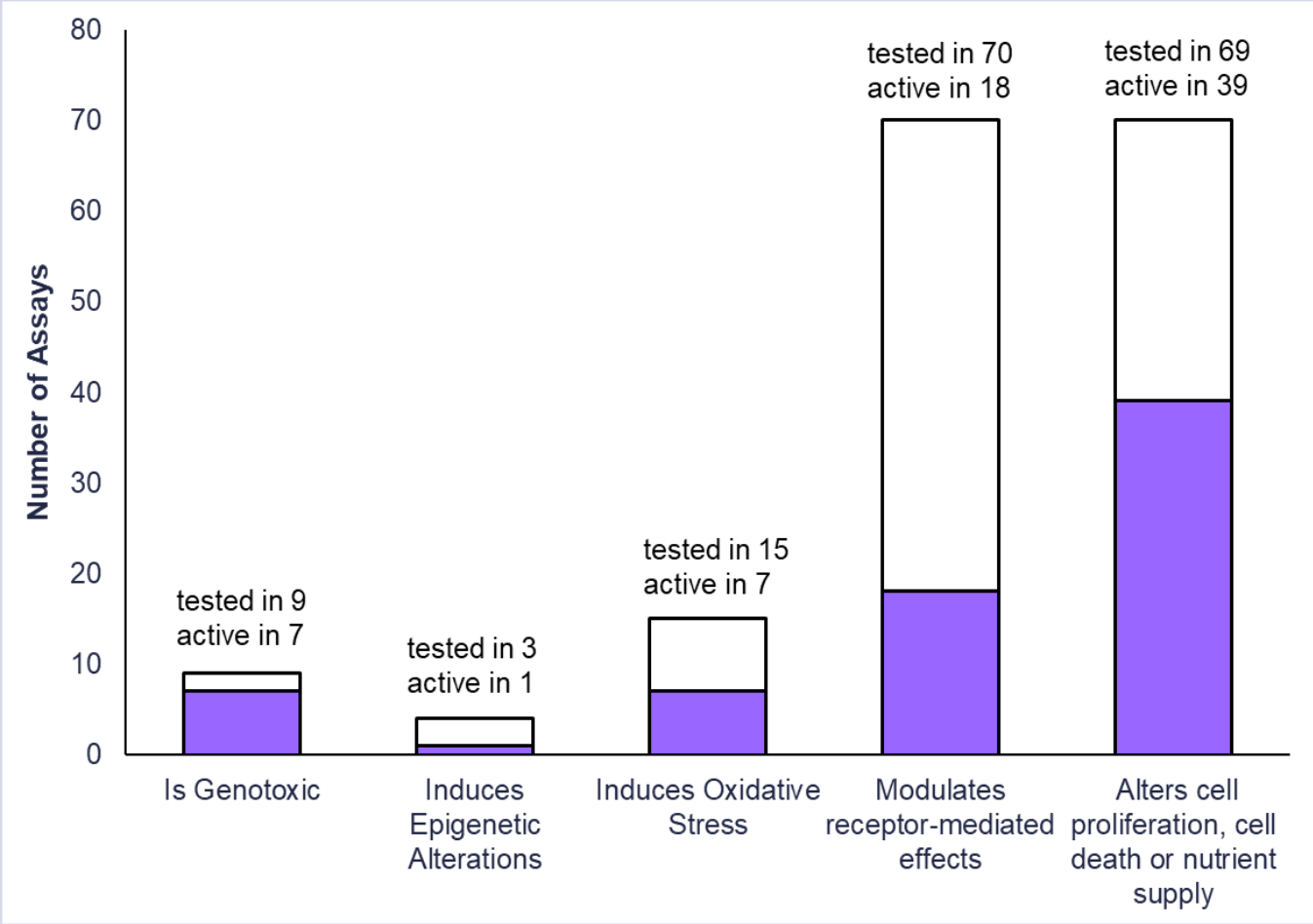
Malachite Green Chloride



Chemicals	Genotoxicity			Animal tumors observed	Common Tumor Sites with Gentian Violet
	Mutagenicity	Chromosomal effects	DNA damage/ DNA binding		
Gentian Violet	+	+	+	Yes	**Hepatocellular, thyroid follicular, testis and epididymis, clitoral gland, Harderian gland, reticulum cell sarcomas (type A), earlier onset of MNCL**
Pentamethyl-pararosaniline chloride	+	NT	+	NT	NT
C.I. Basic Red 9 [1]	+	+	+	Yes	**Hepatocellular Thyroid follicular Harderian gland**
Magenta	+	**NT**	+	No adequate studies	No adequate studies
Malachite Green Chloride	+	+	+	Yes	**(±) Hepatocellular (±) Thyroid follicular**
Leucomalachite Green	+	**NT**	+	Yes	**Hepatocellular (±) Thyroid follicular**
Methyl Green	-	**NT**	-	NT	NT
Michler's Ketone [1]	+	+	+	Yes	**Hepatocellular**

[1] Proposition 65 carcinogen
NT, not tested; MNCL, mononuclear cell leukemia

ToxCast High-Throughput Screening Data for Gentian Violet



- Gentian violet was active in 273/794 ToxCast assays
- These 273 active assays cover 17 biological processes or intended target families
- 72 of the active assays were mapped to IARC's key characteristics of carcinogens

IARC's Key Characteristics of Carcinogens

Key Characteristic [1]	Relevant evidence for gentian violet
1. Is electrophilic or can be metabolically activated	Direct acting electrophile, metabolically activated to nitrogen- and carbon-centered free radicals
2. Is genotoxic	Genotoxicity tests; ToxCast assays
3. Alters DNA repair or causes genomic instability	
4. Induces epigenetic alterations	
5. Induces oxidative stress	Production of reactive oxygen species in cell-free systems; ToxCast assays
6. Induces chronic inflammation	
7. Is immunosuppressive	
8. Modulates receptor-mediated effects	ToxCast assays, including assays on AR, ERα, THRβ
9. Causes immortalization	
10. Alters cell proliferation, cell death, or nutrient supply	

[1]Smith et al. (2016) *Environ Health Perspect*. 2016 Jun; 124(6): 713–721.

Summary: Studies in F344 rats and B6C3F1 mice

*Hepatocellular tumors in M rats (adenoma); M, F mice (adenoma; carcinoma)

*Thyroid follicular tumors in M, F rats (adenocarcinoma; adenoma or adenocarcinoma combined)

*Earlier onset of mononuclear cell leukemia in F rats

*Harderian gland adenomas in M, F mice

*Reticulum cell sarcomas (type A) (likely histiocytic sarcoma) in the bladder, ovaries, uterus, and vagina in F mice

Mesotheliomas of the testis and epididymis in M rats

Clitoral gland tumors in F rats (adenoma or adenocarcinoma combined)

M: male; F: female

* statistically significant

Summary: Other Relevant Data

- **Metabolites include:**
  - C- and N-centered radicals
  - Carcinogens: Formaldehyde, C.I. Basic Red 9, Michler's ketone
  - Additional genotoxic metabolites: pentamethylpararosaniline, N,N,N',N'- and N,N,N',N''- tetramethylpararosaniline, leucogentian violet, and leuco-pentamethylpararosaniline
- **Possible mechanisms of action:**
  - Electrophilicity
    - Direct acting electrophile
    - Forms C- and N- centered radicals during metabolism
  - Induction of oxidative stress
  - Modulation of receptor-mediated effects: AR, ERα, THRβ
  - Genotoxicity
    - Bacterial mutagenicity
    - Chromosomal aberrations in human and mammalian cells
    - Chromosome breakage in human and rodent cells
    - DNA damage in bacteria and mouse lymphocytes
    - DNA binding in multiple systems
    - Genotoxic metabolites
- **Structure activity comparisons:**
  - 6 of 7 comparison chemicals also test positive for genotoxicity
  - 2 are Proposition 65 carcinogens
  - 3 (C.I. Basic Red 9, Michler's ketone, leucomalachite green) also induce liver tumors, and 1 (C.I. Basic Red 9) also induces thyroid and Harderian gland tumors