OFFICE OF ENVIRONMENTAL HEALTH HAZARD ASSESSMENT

Proposition 65

Diaminotoluenes (DATs)

"Diaminotoluene (mixed)" are chemicals listed "as causing cancer" by the authoritative bodies mechanism and are under review by the Carcinogen Identification Committee

August 2015

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

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PREFACE

Proposition 65[1] requires the publication of a list of chemicals that are 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 implementation of Proposition 65[2].

The US Environmental Protection Agency (US EPA) is a designated "authoritative body" whose formal identification of carcinogens serves as the basis for listing chemicals under Proposition 65[3]. Diaminotoluene (mixed) was listed "as causing cancer" under Proposition 65 on January 1, 1990, based upon its classification by US EPA (1988)[4] as a Group B2 carcinogen (probable human carcinogen). On October 21, 2014, OEHHA received a petition from Big Lots Stores, Inc. for reconsideration of the listing for diaminotoluene (mixed). While the US EPA (1988) basis document indicates that the hazard ranking of diaminotoluene (mixed) "is applicable to all isomers of diaminotoluene", and that the "evidence on potential carcinogenicity from animal studies is 'sufficient'", the US EPA document also indicates that "this evidence is based on the carcinogenic properties of the isomer 2,4-diaminotoluene". No other information on the mixture or individual isomers is included in the US EPA document.

OEHHA is referring diaminotoluene (mixed) to the Carcinogen Identification Committee (CIC)[5], the state's qualified experts for carcinogenicity determinations under Proposition 65[6], for a determination as to whether the chemical has been "clearly shown through scientifically valid testing according to generally accepted principles to cause cancer." If the CIC makes such a determination, the chemical remains on the Proposition 65 list. Otherwise, the chemical is removed from the list. The CIC will also determine whether or not diaminotoluenes as a group, or any of the five individual diaminotoluene isomers not currently listed as causing cancer, should be added to the list. 2,4-Diaminotoluene

[1] The Safe Drinking Water and Toxic Enforcement Act of 1986 (California Health and Safety Code 25249.5 *et seq*.)
[2] Health and Safety Code section 25249,12, Title 27, Cal. Code of Regs., section 25102(o)
[3] Title 27, Cal. Code of Regs., section 25306
[4] US Environmental Protection Agency (US EPA,1988). Methodology for Evaluating Potential Carcinogenicity in Support of Reportable Quantity Adjustments Pursuant to CERCLA Section 102. Office of Health and Environmental Assessment, Washington DC 20460, EPA/600/8-89/053, June 1988, including Appendix: Hazard Ranking of Potential Carcinogens, and Appendix: Evaluation of the Potential Carcinogenicity of Diaminotoluene (Mixed) (95-80-7). In Support of Reportable Quantity Adjustments Pursuant to CERCLA Section 102, Carcinogen Assessment Group, Office of Health and Environmental Assessment Washington, DC, 20460 EPA/600/8-91/103 June 1988.
[5] Title 27, Cal. Code of Regs., section 25306(j)
[6] Title 27, Cal. Code of Regs., section 25305(a)(1)

will not be re-considered, as it was individually added to the list by the Science Advisory Panel (the predecessor entity to the CIC) on January 1, 1988.

The public was given the opportunity to submit information relevant to the assessment of the evidence of the carcinogenicity of diaminotoluenes. OEHHA reviewed and considered those submissions in preparing this document.

On November 4, 2015, the CIC is scheduled to deliberate on the carcinogenicity of diaminotoluene (mixed), diaminotoluenes as a group, and the five diaminotoluene isomers not currently listed individually on the Proposition 65 list. OEHHA developed this document with information on the evidence of carcinogenicity of the diaminotoluenes to assist the CIC in its deliberations. The original papers discussed in the document will also be provided to the CIC as part of the hazard identification materials. Comments on this hazard identification document received during the public comment period also form part of the hazard identification materials, and are provided to the CIC members prior to their formal deliberations.

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1. EXECUTIVE SUMMARY

This document summarizes the evidence of carcinogenicity for the chemical group "diaminotoluenes (DATs)". DATs are defined in this document as the chemical group consisting of each of the six DAT isomers: 2,3-DAT, 2,4-DAT, 2,5-DAT, 2,6-DAT, 3,4-DAT, and 3,5-DAT.

"2,4-DAT" and "diaminotoluene (mixed)" have been listed as known to the state to cause cancer under Proposition 65 since January 1, 1988 and January 1, 1990, respectively. 2,4-DAT was listed via the state's qualified experts listing mechanism. "Diaminotoluene (mixed)" was listed via the authoritative bodies listing mechanism, based on its formal identification by the United States Environmental Protection Agency (US EPA) as causing cancer (US EPA, 1988). In classifying "DAT (mixed)" as a Group B2 (probable human carcinogen), US EPA (1988) stated:

> "Diaminotoluene is a probable human carcinogen, classified as weight-of-evidence Group B2 under the EPA Guidelines for Carcinogen Risk Assessment (US EPA, 1986) [US EPA, 1986a]. Evidence on potential carcinogenicity from animal studies is "Sufficient," and the evidence from human studies is 'No Data'." This evidence is based on the carcinogenic properties of the isomer 2,4-diaminotoluene."

No additional data on the carcinogenicity of the mixture or the five other DAT isomers was included in the US EPA document (US EPA, 1988).

OEHHA prepared this document to provide the Carcinogen Identification Committee with information relevant to the assessment of the evidence of carcinogenicity for the following: diaminotoluene (mixed), DATs, 2,3-DAT, 2,5-DAT, 2,6-DAT, 3,4-DAT, and 3,5-DAT.

DATs are a group of synthetic monocyclic aromatic amines. Commercial grades of DATs include a "crude" mix containing all six isomers and two isomer mixes referred to as Meta-DAT and Ortho-DAT. All commercial grades of DATs contain traces of the other isomers (WHO, 1987; Cartolano, 2005).

DATs are used as intermediates or ingredients in the production of a wide variety of industrial and consumer products. The primary use is in the synthesis of toluene diisocyanates. Other uses are as urethane co-reactants, intermediates in the synthesis

of diethyltoluenediamine, tolytriazoles, and mercaptotoluimidazole, as ingredients in dyes for hair, textiles, furs, leather, biological stains, indicators, spirit varnishes, and wood stains, and as an epoxy curing agent and photographic developer (WHO, 1987). Exposure to DATs can occur in a variety of settings, including occupational and non-occupational environments. Industrial workers can be exposed during polyurethane production and the application of permanent hair dyes; consumers can also be exposed through the use of hair dyes, as well as polyurethane-coated breast implants, and potentially via consumption of foods that have been wrapped in commercially available composite food packaging bags.

No studies in humans were identified in the literature specifically designed to investigate the risk of cancer associated with exposure to one or more of the DAT isomers. Several cancer epidemiology studies have been conducted in populations exposed to complex mixtures that contain one or more DAT isomers and that also contain other compounds with known or suspected carcinogenic activity. Populations studied include individuals exposed to 2,4-DAT and/or 2,5-DAT (or its sulfate salt) in hair dye formulations through either occupational or personal use, individuals exposed to mixtures of 2,4-DAT and 2,6-DAT during polyurethane foam production and handling, and individuals with polyurethane-coated breast implants, which may degrade and release 2,4-DAT and 2,6-DAT. Because the exposures in these studies were to mixtures of chemicals (including other carcinogens), and because these studies often lack specific information on 2,4-DAT and/or 2,5-DAT exposure levels, they are very limited in their ability to assess the relationship between exposures to DATs and cancer risk.

The evidence for the carcinogenicity of DATs as a group comes from a substantial body of evidence on 2,4-DAT, together with evidence on four other isomers: 2,3-, 2,5-, 2,6-, and 3,4-DAT.

- 2,3-DAT – observations following treatment include:
    - Mutations in *Salmonella*
    - Positive findings in Bhas 42 cells in the ‘promotion’ cell transformation assay
    - Induction of CYP1A protein expression
    - Findings from ToxCast high-throughput screening (HTS) assays, such as effects on gene expression and transcription factor activities.

- 2,5-DAT – observations following treatment include:
    - Tumor findings
        - In the male F344 rat study by National Cancer Institute (NCI, 1978), the incidence of testicular interstitial cell tumors was significantly increased in the 600 ppm dose group compared to its control group

($p<0.05$), and in the 2000 ppm dose group compared to its control group ($p<0.05$).
        - In the female B6C3F1 mouse study by NCI (1978), the incidences of lung alveolar/bronchiolar adenoma and combined adenoma and carcinoma were significantly increased ($p<0.05$) in the 1000 ppm dose (high dose) group compared to the high-dose control group, but were not signicant in the 600 ppm (low dose) dose group compared to the low-dose control group.
    - Mutations and DNA damage in bacteria
    - Unscheduled DNA synthesis (UDS), DNA damage, and chromosomal aberrations (CA) in mammalian cells
    - DNA damage and inhibition of DNA synthesis in animals
    - Positive findings in *in vitro* cell transformation assays in Syrian hamster embryo (SHE) cells and in Bhas 42 cells in the 'promotion' cell transformation assay

- 2,6-DAT – observations following treatment include:
    - Tumor findings
        - In the male F344 rat study by NCI (1980), significant dose-response trends were observed in the incidences of hepatocellular adenoma ($p<0.05$) and combined adenoma and carcinoma ($p<0.05$).
        - In the male F344 rat study by NCI (1980), a significant dose-response trend was observed in the incidence of pancreatic islet-cell adenoma ($p<0.05$).
        - In the female B6C3F1 mouse study by NCI (1980), a significant dose-response trend was observed in the incidence of hepatocellular carcinoma ($p<0.05$), but not combined adenoma and carcinoma.
    - Mutations and DNA damage in bacteria
    - Chromosomal recombination in yeast
    - Mutations, covalent binding to DNA, UDS, induction of micronuclei (MN), sister chromatid exchanges (SCEs), and CA in mammalian cells
    - DNA damage, DNA adducts, MN, UDS, and inhibition of DNA synthesis in animals
    - In *in vivo* metabolism studies in Sprague-Dawley rats (the only species examined), two mutagenic metabolites, 5-hydroxy-2-acetylamino-6-aminotoluene and 2,6-diacetylaminotoluene, which are mutagenic in *S. typhimurium*, were identified
    - Weakly positive in one *in vitro* SHE cell transformation assay

- 3,4-DAT – observations following treatment include:
    - Mutations in bacteria (weakly positive)
    - Inhibition of DNA synthesis in animals
    - Weakly positive in one *in vitro* cell transformation assay in SHE cells and positive in Bhas 42 cells in the 'promotion' cell transformation assay
    - Findings from ToxCast HTS assays, such as effects on gene expression and transcription factor activities

All the isomers bear close structural similarity to 2,4-DAT, which is already listed under Proposition 65 and not under reconsideration. The findings for 2,4-DAT are:

- Tumor findings:
    - Alveolar bronchiolar carcinomas in male B6C3F1 mice
    - Bone osteosarcoma in female Fischer rats
    - Liver tumors in male and female Fischer rats, male CD rats, male Wistar rats, female CD-1 mice, and female B6C3F1 mice
    - Lymphomas in female B6C3F1 mice
    - Mammary gland tumors in male and female Fischer rats
    - Subcutaneous fibromas in male and female Fischer rats and male CD rats
    - Subcutaneous sarcomas in male and female rats (strain not specified)
    - Vascular tumors in male and female CD-1 mice
- One mutagenic metabolite, 4-acetylamino-2-aminotoluene, was identified in rats
- DNA adducts *in vivo* and genotoxic in bacteria, mammalian cells, and animals
- Induction of cell transformation *in vitro*, and cell proliferation in rat liver *in vivo*
- Altering the expression of cell cycle and apoptosis related genes, induction of CYP1A protein
- Findings from ToxCast HTS assays, including activation of receptors such as aryl hydrocarbon receptor (AhR), androgen receptor (AR), estrogen receptor (ER), and peroxisome proliferator-activated receptors (PPARs)

Other relevant data supporting the carcinogenicity of DATs come from comparison of biological activities amongst the DAT isomers and with four structurally related chemicals. The findings are:

- All of the DAT isomers except 3,5-DAT have been tested and found to be active in genotoxicity assays, as have each of the four comparison monocyclic aromatic amines (with the exception of *p*-cresidine, which has not been tested for *in vitro* cell transformation).
    - In comparing the genotoxic activity of 2,4-DAT with that of the other DAT isomers:

        - 2,3-, 2,5-, 2,6-, and 3,4-DAT[7] were also each mutagenic
        - 2,5- and 2,6-DAT also induced chromosomal effects
        - 2,5-, 2,6- and 3,4-DAT also induced DNA damage or other DNA effects
- All of the DAT isomers except 3,5-DAT have been tested and found to be active in *in vitro* cell transformation assays, as have each of the four comparison monocyclic aromatic amines (with the exception of *p*-cresidine, which has not been tested).
- Despite the limitations in the conduct and design of the animal cancer bioassays on 2,5-DAT and 2,6-DAT, increases in the incidence of tumors were observed for these two DAT isomers at sites in common with 2,4-DAT target tumor sites (2,5-DAT: lung tumors in mice, 2,6-DAT: liver tumors in rats and mice).
- While not all the quantitative structure activity relationship models run were in agreement, both the VEGA (with the exception of the carcinogenicity prediction for 3,5-DAT) and Lazar models predicted 2,3-, 2,5-, 2,6-, 3,4- and 3,5-DAT to be mutagenic and carcinogenic.

[7] 3,4-DAT was weakly positive in one study in *Salmonella*

2. INTRODUCTION

This document summarizes the evidence of carcinogenicity for the chemical group "diaminotoluenes (DATs)", defined in this document as consisting of each of the six DAT isomers. These six isomers are 2,3-DAT, 2,4-DAT, 2,5-DAT, 2,6-DAT, 3,4-DAT, and 3,5-DAT (see Table 1).

"2,4-DAT" and "diaminotoluene (mixed)" have been listed as known to the State to cause cancer under Proposition 65 since January 1, 1988 and January 1, 1990, respectively. 2,4-DAT was listed via the state's qualified experts listing mechanism. "Diaminotoluene (mixed)" was listed via the authoritative bodies listing mechanism, based on formal identification by the United States Environmental Protection Agency (US EPA) as causing cancer (US EPA, 1988). In classifying "DAT (mixed)" as a Group B2 (probable human carcinogen), US EPA stated:

> "Diaminotoluene is a probable human carcinogen, classified as weight-of-evidence Group B2 under the EPA Guidelines for Carcinogen Risk Assessment (US EPA, 1986) [US EPA, 1986a]. Evidence on potential carcinogenicity from animal studies is "Sufficient," and the evidence from human studies is 'No Data'." This evidence is based on the carcinogenic properties of the isomer 2,4-diaminotoluene." (US EPA, 1988)

No additional data on the carcinogenicity of the mixture or the five other DAT isomers was included in the US EPA document (US EPA, 1988).

On October 21, 2014, OEHHA received a petition from Big Lots Stores, Inc. for reconsideration of the listing for diaminotoluene (mixed).

OEHHA is referring diaminotoluene (mixed) to the Carcinogen Identification Committee (CIC)[8], the state's qualified experts for carcinogenicity determinations under Proposition 65[9], for a determination as to whether the chemicals as a group or individually have been "clearly shown through scientifically valid testing according to generally accepted principles to cause cancer."

[8] Title 27, Cal. Code of Regs., section 25306(j)
[9] Title 27, Cal. Code of Regs., section 25305(a)(1)

OEHHA prepared this document to provide the CIC with information relevant to the assessment of the evidence of carcinogenicity for the following: diaminotoluene (mixed), DATs as a group, 2,3-DAT, 2,5-DAT, 2,6-DAT, 3,4-DAT, and 3,5-DAT.

Carcinogenicity evidence from studies of 2,4-DAT, which is listed separately as known to the state to cause cancer, has been included in this document as information relevant to the assessment of diaminotoluene (mixed) and DATs, and for structure-activity comparison purposes with the other five DAT isomers.

2.1 Identity of Diaminotoluenes (DATs)

DATs are a group of synthetic monocyclic aromatic chemicals constituted of two primary amino groups and a methyl group attached to a benzene ring. The molecular weight of each DAT is 122.17 grams per mole (g/mol) and the molecular formula is $C_7H_{10}N_2$ (ChemIDPlus, 2015). The purified DAT isomers are colorless crystals, while the commercial DAT mixtures (depending on their isomeric compositions) are soilds of varied colors: light yellow to tan, or light grey to purple. Both the pure DAT isomers and the commercial mixtures are soluble in hot water, alcohol, ether, and hot benzene (WHO, 1987).

Figure 1 shows the numeric labeling scheme used for the two amino ($-NH_2$) groups in naming the different DAT isomers. Table 1 shows some chemical and physical properties of the six DAT isomers as well as the systematic names and synonyms.

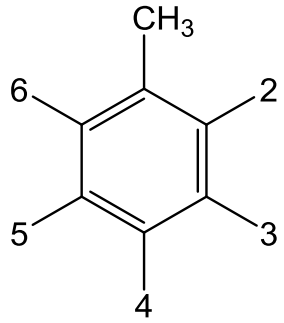
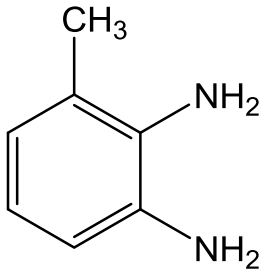
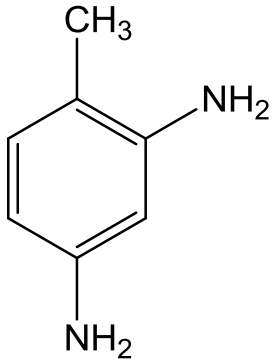
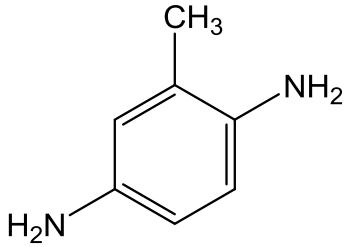
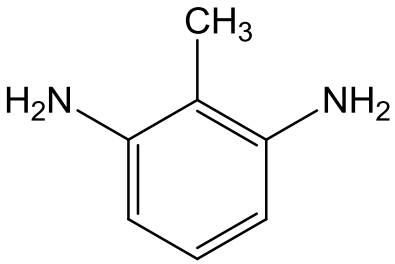
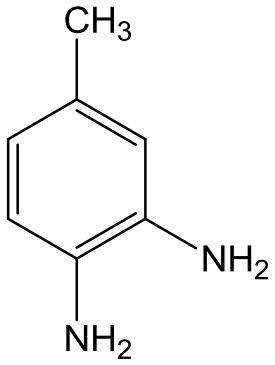


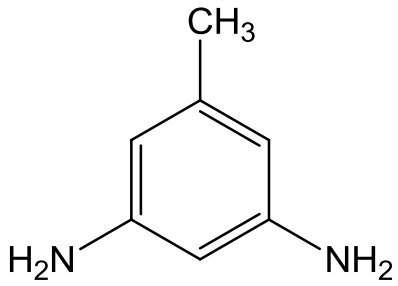
Figure 1. DAT numeric labeling scheme for the $-NH_2$ groups

Table 1. Identity and selected chemical and physical properties of the DAT isomers

Chemical Name & Synonyms[1]	CAS #	Structure	Solubility in water at 25°C (mg/L)	Log K_{ow} (octanol:water partition coefficient)	Melting point (°C)
2,3-DAT 1,2-Benzenediamine, 3-methyl- 1,2-Diamino-3-methylbenzene 1-Methyl-2,3-phenylenediamine 2,3-Toluylenediamine 2,3-Tolylenediamine 3-Methyl-1,2-benzenediamine 3-Methyl-1,2-phenylenediamine 3-Methyl-o-phenylenediamine Toluene, 2,3-diamino- 2,3-toluenediamine toluene-2,3-diamine	2687-25-4		2.46×10^4	0.710	63.5
2,4-DAT[2] 1,3-Diamino-4-methylbenzene 2,4-Diamino-1-methylbenzene 2,4-Diamino-1-toluene 2,4-Diaminotoluen 2,4-Diaminotoluen [Czech] 2,4-Diaminotoluene 2,4-Diaminotoluol 2,4-Tolamine 2,4-Toluenediamine 3-Amino-p-toluidine 4-m-Tolylenediamine	95-80-7		7.48×10^4	0.14	99

Chemical Name & Synonyms[1]	CAS #	Structure	Solubility in water at 25°C (mg/L)	Log K_{ow} (octanol:water partition coefficient)	Melting point (°C)
4-Methyl-1,3-benzenediamine 4-Methyl-m-phenylenediamine 5-Amino-o-toluidine m-Toluenediamine m-Toluylendiamin m-Tolylenediamine meta-Tolylenediamine					
2,5-DAT 1,4-Benzenediamine, 2-methyl- 2,5-Diaminotoluene 2-Methyl-1,4-benzenediamine 2-Methyl-p-phenylenediamine 2-Methyl-para-phenylenediamine 4-Amino-2-methylaniline p,m-Tolylenediamine p-Toluenediamine p-Toluylendiamine para-Toluenediamine para-Toluylenediamine para-Tolylenediamine Toluene-2,5-diamine Toluylene-2,5-diamine	95-70-5		7.72 x10^4	0.16	64

Chemical Name & Synonyms[1]	CAS #	Structure	Solubility in water at 25°C (mg/L)	Log K_{ow} (octanol:water partition coefficient)	Melting point (°C)
2,6-DAT 1,3-Benzenediamine, 2-methyl- 2,6-Diamino-1-methylbenzene 2,6-Diaminotoluene 2,6-Toluylenediamine 2,6-Tolylenediamine 2-Methyl-1,3-benzenediamine 2-Methyl-1,3-phenylenediamine 2-Methyl-m-phenylenediamine o-Toluene diamine Toluene, 2,6-diamino- Toluene-2,6-diamine	823-40-5		7.25 x10^4	0.16	106
3,4-DAT 1,2-Benzenediamine, 4-methyl- 1,2-Diamino-4-methylbenzene 3,4-Diamino-1-methylbenzene 3,4-Diaminotoluene 3,4-Toluenediamine 3,4-Toluylenediamine 3,4-Tolylenediamine 4-Methyl-1,2-benzenediamine 4-Methyl-1,2-diaminobenzene 4-Methyl-1,2-phenylenediamine 4-Methyl-o-phenylenediamine	496-72-0		2.69 x10^4	0.66	89.5

Chemical Name & Synonyms[1]	CAS #	Structure	Solubility in water at 25°C (mg/L)	Log K_{ow} (octanol:water partition coefficient)	Melting point (°C)
3,5-DAT toluene-3,5-diamine 5-methylbenzene-1,3-diamine 1,3-Benzenediamine, 5-methyl- 1,3-Diamino-5-methylbenzene 3,5-diaminotoluene 5-Methyl-1,3-benzenediamine 5-Methyl-1,3-benzoldiamin 5-Methylbenzene-1,3-diamine 1,3-Benzenediamine, ar-methyl-	26346-38-3, 26764-44-3 & 108-71-4		7.25 x10^4	0.337	67.3[3] (predicted)

[1] Information obtained from ChemIDPlus (2015) and ChemSpider (2015)

[2] Listed individually under Proposition 65 as causing cancer

[3] Predicted melting point generated using the US EPA's Estimation Program Interface Suite™, according to ChemSpider (2015)

2.2 Occurrence, Use, and Exposure

2.2.1 Production, Use, and Occurrence

DATs are commercially synthesized via catalytic hydrogenation of dinitrotoluenes (DNTs) or by reduction of DNT in the presence of iron and hydrochloric acid (WHO, 1987). Commercial grades of DATs include a "crude' mix containing all six isomers; an isomer mix referred to as Meta-DAT containing either 80 percent 2,4-DAT and 20 percent 2,6-DAT, or 65 percent 2,4-DAT and 35 percent 2,6-DAT; and an isomer mix referred to as Ortho-DAT containing 60 percent 3,4-DAT and 40 percent 2,3-DAT. The 2,5-DAT isomer and its salt are also marketed. 2,3- and 3,5-DAT are formed as minor by-products during the industrial manufacture of other DAT isomers. All commercial grades of DATs contain traces of the other isomers (WHO, 1987; Cartolano, 2005).

DATs are used as intermediates in the production of a wide variety of industrial and consumer products. Over 90% of all uses of DATs are in the synthesis of toluene diisocyanates (TDI) (WHO, 1987). Other uses are as urethane co-reactants, intermediates in the synthesis of other industrial chemicals (diethyltoluenediamine, tolytriazoles, mercaptotoluimidazole), and ingredients in dyes for hair, textiles, furs, leather, biological stains, indicators, spirit varnishes, and wood stains (WHO, 1987). Some limited uses of DATs are as an epoxy curing agent and photographic developer (WHO, 1987). Production and use information for specific DAT mixtures and DAT isomers is briefly summarized below, followed by information on the environmental occurrence of DATs.

Ortho-DAT and Meta-DAT mixtures are used as co-reactants or as raw materials in the manufacture of urethane products, corrosion inhibitors, and antioxidants in nitrile rubber elastomers. Meta-DAT is also used to produce diethyltoluenediamine for urethane elastomer production (WHO, 1987).

2,3-DAT, as part of the Ortho-DAT mixture, is used as a urethane co-reactant, and an intermediate in the production of industrial chemicals such as tolyltriazole and mercaptotoluimidazole (WHO, 1987). In 2002, the production plus import volume of 2,3-DAT was between 1 to 10 million pounds (lbs) (US EPA, 2002).

2,4-DAT is the most important industrial isomer and has been produced in the US since 1919 (NTP, 2014a). Its primary uses are in the production of TDI and dyes. Other uses include the manufacture of impact resins, polyamides, antioxidants, hydraulic fluids, urethane foams, fungicide stabilizers, and photographic developers. Production plus

imports of 2,4-DAT totaled 100 to 500 million lbs up until 1994. As of 2002, the amount of reported quantities decreased to between 10,000 to 500,000 lbs (US EPA, 2002).

2,5-DAT and its sulfate salt (2,5-DAT sulfate) are some of the most widely used ingredients in hair dyes. The isomer or its salt is present in about 20 percent of the commercial hair dyes in the US (Kinkel and Holzmann, 1973; Gube *et al.*, 2011; Hamann *et al.*, 2014). In 2005, approximately 150 to 200 metric tons of 2,5-DAT was used as an oxidative hair dye ingredient worldwide (IARC, 2010). There is no report on the production volume of this isomer in the US (US EPA, 2002).

2,6-DAT, as part of the Meta-DAT mixture, is used in the synthesis of TDI. It is also an intermediate used in the synthesis of several dyes used for textiles and furs. In 2002, the reported production plus imports of 2,6-DAT in the US was between 10,000 to 500,000 lbs (US EPA, 2002).

3,4-DAT is present in the Ortho-DAT mixture. Like 2,3-DAT, uses of 3,4-DAT include as a urethane co-reactant, in epoxy curing, and as an intermediate for tolyltriazole and mercaptotoluimidazole synthesis (WHO, 1987). The production plus import volume was between 1 to 10 million lbs in the US in 2002 (US EPA, 2002).

There is no known use of the pure 3,5-DAT isomer. It is found as a minor by-product of the synthesis of other DAT isomers (Milligan and Gilbert, 1978; WHO, 1987).

DATs may be released to the environment during manufacturing or transportation of the chemicals or through industrial wastes deposited in landfills (WHO, 1987; Kim *et al.*, 2002). An estimated 6000 metric tons of DATs are disposed into authorized landfills, and roughly 1400 metric tons of DATs are released to the environment during the production of the chemicals (WHO, 1987). More recent data from the US EPA Toxics Release Inventory database (accessible via TOXNET at http://toxnet.nlm.nih.gov/) showed that 3,967 lbs of DATs were released into the environment in 2013, with the vast majority being released into the air. Additionally, total off-site waste transfer amounted to 8,168,463 lbs in the same year.

DATs may form in the environment via biodegradation of DNTs, which are used in the manufacture of explosives, propellants, pesticides, and dyes (Yang *et al.*, 2008). Due to the high water solubility of DATs, their leakage from landfills or storage sites and accidental spillage during shipping and handling may lead to surface and groundwater contamination (WHO, 1987).

2.2.2 Exposure

Human exposure to DATs can occur in a variety of settings, including occupational and non-occupational environments. For example, exposure can occur through inhalation and dermal contact in occupational settings where DATs are produced or used as intermediates, or where DAT-containing products are manufactured or used. A World Health Organization (WHO) report (WHO, 1987) notes that workers can be exposed to DATs at air concentrations of up to 0.44 milligram per cubic meter (mg/m^3), and occasionally to levels reaching 11 mg/m^3. Exposure information on specific DATs or for specific pathways is briefly summarized below.

In a polyurethane foam processing plant, concentrations of 2,4-DAT and 2,6-DAT were detected ranging from 2 to 6.5 parts per million (ppm) and 65 to 110 ppm, respectively, in areas near the demolding step of the manufacturing process (Lewandowski *et al.*, 2005).

2,4- and 2,6-DAT have been detected in the urine and plasma of workers who are exposed to TDI, and measurements of these DATs in the urine or plasma can serve as biomarkers of occupational exposure to TDI (Brorson *et al.*, 1991; Lind *et al.*, 1996; Dalene *et al.*, 1997; Kaaria *et al.*, 2001).

Exposure to 2,4- and 2,6-DAT may occur in patients with existing breast implants that contain polyurethane (polyurethane foam covers are no longer approved by US Food and Drug Administration [US FDA] for use in breast implants). The polyurethane foam cover of breast implants contains a mixture of 2,4- and 2,6-TDI, which can break down and form 2,4- and 2,6-DAT, respectively (Batich *et al.*, 1989; Luu and White, 1993). Animal studies have shown that these polyurethane foam-covered implants provoke a foreign body reaction, which leads to degradation of the polyurethane foam and production of 2,4- and 2,6-DAT within 6 to 12 months post-implantation (Picha *et al.*, 1990). These findings in animals are consistent with clinical observations made in patients (Smahel, 1978; Picha *et al.*, 1990).

Consumers can potentially be exposed to 2,4-DAT via consumption of foods that have been packaged in commercially available composite laminated food packaging bags (e.g., used for corn chips and frozen foods). TDI is used as a curing agent in these composite food packaging bags, and 2,4-DAT can be formed via hydrolysis of TDI. Trace amounts of 2,4-DAT were detected in migration tests on composite food packaging bags using water as a food simulant, including bags used for chicken wings, brown sugar, oatmeal, and rice (Wang and Chen, 2009; Zhang, 2010).

Occupational and consumer exposures to 2,5-DAT occur during application of permanent hair dyes or tints (Gube *et al.*, 2011). About 21 percent of the commercial hair dyes in the US contain 2,5-DAT (Hamann *et al.*, 2014). A survey of the cosmetics industry cited by Burnett *et al.* (2010) states that hair-dye products contain 2,5-DAT at concentrations ranging from 0.2 to 3.0 percent in hair dyes and colors, and 2,5-DAT sulfate at concentrations from 0.1 to 4.0 percent in hair dyes and colors and 3 percent in hair bleaches. A study of hairdressers and personal users of hair dye found that the urinary levels of 2,5-DAT were approximately 200-fold higher in personal users compared to hairdressers (Gube *et al.*, 2011). No detectable levels of 2,5-DAT were found in air sampled from local exhaust ventilation in hair salons (Hollund and Moen, 1998), demonstrating that dermal contact with hair dyes is probably the most predominant route of occupational exposure to 2,5-DAT in hairdressers.

3. DATA ON CARCINOGENICITY

3.1 Carcinogenicity Studies in Humans

No studies in humans were identified in the literature specifically designed to investigate the risk of cancer associated with exposure to one or more of the DAT isomers. Several cancer epidemiology studies have been conducted in populations exposed to complex mixtures that may contain one or more DAT isomers and that also contain other compounds with known or suspected carcinogenic activity. These populations include individuals exposed to 2,4-DAT and/or 2,5-DAT (or its sulfate salt) in hair dye formulations through either occupational or personal use, individuals exposed to mixtures of 2,4-DAT and 2,6-DAT during polyurethane foam production and handling, and individuals with polyurethane-coated breast implants, which may degrade and release 2,4-DAT and 2,6-DAT.

With regard to hair dye exposures, multiple carcinogens have historically been present in some hair dye formulations (*e.g.*, 2,4-diaminoanisole, 4-chloro-*m*-phenylenediamine, 2-nitro-*p*-phenylenediamine, 4-amino-2-nitrophenol) (IARC, 2010), and the available epidemiology studies of occupational and personal exposure to hair dyes provide little or no information on cancer risks associated specifically with exposure to the DATs. In 2010 the International Agency for Research on Cancer (IARC) published a comprehensive review on the cancer risk from occupational exposure of hairdressers and barbers and personal use of hair dye that concluded there is "*limited evidence* in humans for the carcinogenicity of occupational exposures as a hairdresser or barber", and "*inadequate evidence* in humans for the carcinogenicity of personal use of hair

colorants" (emphasis in the original) (IARC, 2010). The 2010 IARC review is included here as an Attachment. Brief summaries of cancer epidemiology studies of populations exposed to hair dyes published since the 2010 IARC review are included in Appendix A.

With regard to chemical exposures during the production and handling of polyurethane foam, these include not only 2,4-DAT and 2,6-DAT, which are synthesis byproducts and foam degradation products, but also the carcinogen TDI, which is used as a starting material in the manufacture of polyurethane foam. From the available cancer epidemiology studies of polyurethane foam industry workers, it is not possible to differentiate between the risks associated with exposures to TDI and the DATs, and thus these studies provide little insight on DAT-related cancer risks. These studies are briefly summarized in Appendix A.

Polyurethane-coated breast implants, which have been shown to release 2,4-DAT and 2,6-DAT, have not been used in the US since 1991 (Castel *et al.*, 2015; FDA, 2015). Three studies were identified that compared risks of breast cancer in women with polyurethane-coated implants to risks in women with non-polyurethane-coated implants (Brisson *et al.*, 2006; Lavigne *et al.*, 2012; Pan *et al.*, 2012). Brisson *et al.* (2006) reported a significantly increased risk of breast cancer associated with one type of polyurethane-coated implants, namely subglandular breast implants. Risk was highest during the first five years after surgery, and then declined with time since surgery. Neither Lavigne *et al*. (2012) nor Pan *et al.* (2012) reported any significantly increased risks associated with polyurethane-coated implants. These studies are briefly summarized in Appendix A.

3.2 Carcinogenicity Studies in Animals

Office of Environmental Health Hazard Assessment (OEHHA) conducted a literature search on each of the DAT isomers, and identified animal carcinogenicity studies conducted on 2,4-DAT, 2,5-DAT and 2,6-DAT. Each of the three isomers has been studied in rats and mice. OEHHA was unable to identify any carcinogenicity studies on 2,3-, 3,4-, or 3,5-DAT. Findings from studies on 2,4-DAT, which is already listed individually as causing cancer under Proposition 65, are summarized in Section 3.2.1. Studies on 2,5- and 2,6-DAT are discussed in Sections 3.2.2 and 3.2.3, respectively.

OEHHA also identified studies of complex mixtures containing several compounds, including one or more DAT isomers. These studies have limited usefulness for the cancer hazard identification of DATs, and are briefly discussed in Section 3.2.4.

3.2.1 2,4-DAT

The available animal cancer bioassays of 2,4-DAT consist of four subcutaneous studies in rats, four dietary studies in rats, and four dietary studies in mice. Table 2 presents a summary of the tumor findings for 2,4-DAT from these 12 studies.

Table 2. Summary of tumor findings for 2,4-DAT

Species/ Route of Administration	Strain	Sex[1]	Tumor Types	References
Rat, subcutaneous injection	Not Specified	M	Subcutaneous sarcoma[2]	Umeda (1955), as reviewed in IARC (1978)
		F	Subcutaneous sarcoma[2]	
	Wistar	M	Hepatocellular carcinoma	
	CD	M	Subcutaneous fibroma Liver tumors[3]	Weisburger *et al.* (1978)
Rat, diet	Fischer	M	Hepatocellular tumors (benign and malignant) Mammary tumors[3] (fibroadenoma, adenoma, carcinosarcoma, and adenocarcinoma)	Cardy (1979)
		F	Hepatocellular tumors (benign and malignant) Mammary tumors (fibroadenoma, adenoma, carcinosarcoma, and adenocarcinoma) Bone osteosarcoma[4]	
		M	Hepatocellular neoplastic nodule and carcinoma; Mammary gland adenoma[4] Subcutaneous fibroma	NCI (1979)
		F	Hepatocellular neoplastic nodule and carcinoma Mammary adenoma and carcinoma Subcutaneous fibroma	
Mouse, diet	CD-1	M	Vascular tumors[2]	Weisburger *et al.* (1978)
		F	Liver hepatoma Vascular tumors	
	B6C3F1	M	Alveolar bronchiolar carcinoma	NCI (1979)
		F	Hepatocellular carcinoma Lymphoma	

[1] M: male; F: female
[2] Tumors were observed at injection sites
[3] Statistically significant compared to pooled controls
[4] Biologically significant – rare tumor (Goodman *et al.*, 1979)

3.2.2 2,5-DAT

OEHHA identified one National Cancer Institue (NCI) report on the carcinogenicity of 2,5-DAT sulfate in animals (NCI, 1978). The NCI report included four dietary bioassays, one each in male and female F344 rats, and one each in male and female B6C3F1 mice.

3.2.2.1 Long-term 2,5-DAT sulfate feeding studies in male and female Fischer 344 rats (NCI, 1978)

Under the direction of NCI, the Mason Research Institute in Worcester, MA conducted two carcinogenicity studies of 2,5-DAT sulfate, one in male Fischer 344 (F344) rats and one in female F344 rats (NCI, 1978). In each study, 2,5-DAT sulfate (99 percent purity) was administered to groups of 50 rats in the diet at initial concentrations of 300 ppm (0.03 percent) or 500 ppm (0.05 percent) in the feed. In both studies, each of the treatment groups had its own control group of 50 animals fed control diet. Animals were six weeks old at initiation of the studies.

Since the researchers found no observable mean body weight depression or clinical signs of toxicity in the 500 ppm groups in either study, all animals in the 300 ppm groups in each study and their corresponding controls were terminated two months after study initiation. In each study, the concentration of 2,5-DAT sulfate was increased in the feed of the 500 ppm treatment groups to 600 ppm (0.06 percent) from week 14 to 78. After treatment ended at week 78, animals in these groups were observed for an additional 28 weeks (males) or 29 weeks (females) before termination. These dose groups were designated the “low-dose” groups in the final evaluation of each study.

An additional treatment group of 50 animals, designated as the “high-dose” group, and a concurrent control group of 25 animals were introduced into each study 11 months after study initiation. Animals in the high-dose group in each study received 2,000 ppm (0.2 percent) of 2,5-DAT sulfate in the diet for 78 weeks, followed by an observation period of 30 weeks (male) or 31 weeks (female) before termination. The dosed groups and their respective controls were housed in separate rooms during the study. Overall, the study authors considered the time-weighted-average 2,5-DAT sulfate concentration levels in the feed of the low-dose and the high-dose groups in these studies to be 600 ppm and 2000 ppm, respectively.

Male

In the study in male rats, survival in each treatment group exceeded that in the concurrent control group. Eighty-four percent (42/50) of the low dose, 58 percent

(29/50) of the low-dose control, 90 percent (45/50) of the high dose, and 72 percent (18/25) of the high-dose control survived to at least 85 weeks. The study authors determined that "Adequate numbers of animals in all groups survived sufficiently long to be at risk from late-developing tumors." No change in body weight was observed in either treatment group compared to concurrent controls.

Significantly increased incidences of testicular interstitial cell tumors were observed in both the low-dose and high-dose groups, compared to their respective controls (Table 3). The study authors discounted these tumors based on their high spontaneous incidence in rats, as reported in the literature (Cockrell and Garner, 1976). While it is true that male F344 rats have a high spontaneous incidence of testicular interstitial cell tumors, e.g., 80.5 percent (1445/1794) as reported by Goodman *et al.* (1979) in NCI Carcinogenesis Testing Program studies conducted from 1972 to 1978, the statistically significant increases observed in this study in both dose groups, as compared to their respective controls, suggest that these tumors are treatment-related.

Table 3. Tumor incidence[1] in male F344 rats fed diets containing 2,5-DAT sulfate for 78 weeks and observed for 28 weeks (600 ppm) or 30 weeks (2,000 ppm) (NCI, 1978)

Tumor Site and Type	Time-weighted-average concentration (ppm)			
	control	600	control	2,000
Testicular interstitial cell tumors (%)	33/45 (73%)	43/48* (90%)	19/24 (79%)	47/48* (98%)

* $p<0.05$ compared to the corresponding control by Fisher pairwise test
[1]Tumor incidences are given as the ratio of the number of tumor-bearing animals at a specific anatomic site (numerator) to the number of animals that are still alive at the time of first occurrence of this tumor (denominator).

Female

In the study in female rats, no difference in survival was observed between the low-dose group and concurrent (low-dose) controls. Survival in the high-dose group exceeded that observed in concurrent (high-dose) controls. Eighty-four percent (42/50) of the low dose, 66 percent (33/50) of the low-dose control, 86 percent (43/50) of the high dose, and 68 percent (17/25) of the high-dose control survived to at least 85 weeks. The study authors determined that "Adequate numbers of animals in all groups survived sufficiently long to be at risk from late-developing tumors." The high-dose female rats exhibited a reduction in mean body weight (as compared to controls) of at least 11 percent from week 20 onwards. No treatment-related increases in the incidence of tumors were reported in this study.

3.2.2.2 Long-term 2,5-DAT sulfate feeding studies in male and female B6C3F1 mice (NCI, 1978)

Under the direction of NCI, the Mason Research Institute in Worcester, MA conducted two carcinogenicity studies of 2,5-DAT sulfate, one in male B6C3F1 mice and one in female B6C3F1 mice (NCI, 1978). In each study, 2,5-DAT sulfate (99 percent purity) was administered to groups of 50 mice at initial concentrations of 300 ppm (0.03 percent) and 600 ppm (0.06 percent) in the feed.

Due to the lack of observed mean body weight depression or other clinical signs of toxicity in either study, the 300 ppm treatment groups in each study were discontinued six months after study initiation. The 600 ppm treatment groups were designated as the "low-dose" groups in the final evaluation of each study. An additional treatment group of 50 animals receiving 1,000 ppm (0.1 percent) 2,5-DAT sulfate in the feed was introduced into each study six months after study initiation, and designated as the high-dose" group.

Separate groups of non-concurrent controls (50 animals/group) were included for each treatment group in each study. These controls were received in separate shipments from their respective treatment groups. In each study, low-dose controls were placed on test two weeks after the low-dose treatment groups, while high-dose controls were placed on test two months before the high-dose treatment groups. The high-dose control groups were housed in different rooms than the high-dose treatment groups.

All treated animals in each study received 2,5-DAT sulfate in the diet for 78 weeks. The low-dose treatment groups were observed for an additional 16 weeks and the high-dose treatment groups were observed for an additional 19 weeks, before termination at 94 and 97 weeks, respectively. In the male study, the low-dose controls were sacrificed at 96 weeks and the high-dose controls at 98 weeks. In the female study, the low-dose controls were sacrificed at 97 weeks and the high-dose controls at 98 weeks.

Male

In the study in male mice, no treatment-related differences in body weight or survival were observed between each treatment group and its respective control. Five mice each from the low-dose control, high-dose treatment, and high-dose control groups were sacrificed in week 78. Survival at the end of the study was 94 percent (47/50) in the low-dose treatment group, 84 percent (42/50) in the low-dose controls, 74 percent (37/50) in the high-dose treatment group, and 74 percent (37/50) in the high-dose controls. The study authors determined that "Adequate numbers of animals in all

groups survived sufficiently long to be at risk from late-developing tumors."

The incidence of hepatocellular carcinomas was elevated but did not reach statistical significance (pairwise comparison, p=0.18) in the high-dose treatment group as compared to the high-dose control. Hepatocellular carcinoma incidence was as follows: low-dose control (7/48), low dose (8/48), high-dose control (10/45), and high dose (16/49).

Female

In the study in female mice, the high-dose treatment group showed mean body weight depression, compared to its control group. However, the report states that the growth pattern (i.e., body weight gain) of the control group was unusual in that it did not level off as the mice approached maturity. No treatment-related differences in survival were observed between each treatment group and its respective control. Five mice each from the low-dose control, high-dose treatment, and high-dose control groups were sacrificed in week 78. Survival at the end of the study was 78 percent (39/50) in the low-dose treatment group, 74 percent (37/50) in the low-dose controls, 66 percent (33/50) in the high-dose treatment group, and 70 percent (35/50) in the high-dose controls. The study authors determined that "Adequate numbers of animals in all groups survived sufficiently long to be at risk from late-developing tumors."

Statistically significant increases in the incidences of alveolar/bronchiolar adenomas and combined alveolar/bronchiolar adenomas and carcinomas were observed in the high-dose group compared to the high-dose controls (Table 4). The incidence of alveolar/bronchiolar tumors observed in the high-dose control (1/44, 2.3 percent) is similar to the spontaneous incidence of alveolar/bronchiolar tumors (i.e., combined alveolar/bronchiolar adenoma and carcinoma, alveolar-cell adenocarcinoma, and adenoma not otherwise specified) reported in female B6C3F1 mice from the same laboratory (12/350, 3 percent) (NCI, 1978).

Table 4. Tumor incidence[1] in female B6C3F1 mice fed diets containing 2,5-DAT sulfate for 78 weeks and observed for an additional 16 weeks (600 ppm) or 19 weeks (1,000 ppm) (NCI, 1978)

Tumor Site and Type		Time-weighted-average concentration (ppm)			
		control	600	control	1,000
Lung	Alveolar/bronchiolar adenoma	3/45 (6.7%)	6/42 (14.3%)	1/44 (2.3%)	7/45* (15.6%)
	Alveolar/bronchiolar carcinoma	1/45 (2.2%)	0/42	0/44	1/45 (2.2%)
	Combined	4/45 (8.9%)	6/42 (14.3%)	1/44 (2.3%)	8/45***** (17.8%)

* p<0.05 compared to the corresponding control by Fisher pairwise test, conducted by OEHHA.

[1]Tumor incidences are given as the ratio of the number of tumor-bearing animals at a specific anatomic site (numerator) to the number of animals that are still alive at the time of first occurrence of this tumor (denominator).

3.2.2.3 Conclusions and peer-review comments on the 1978 NCI 2,5-DAT sulfate studies in male and female F344 rats and B6C3F1 mice

The study authors estimated the 95 percent confidence intervals on the relative cancer risk, based on the observed tumor incidence rates in the mouse studies, and stated:

> "It should be noted that many of the confidence intervals have an upper limit greater than one, indicating the theoretical possibility of tumor induction in mice by 2,5-toluenedamine [2,5-DAT] sulfate that could not be established under the condition of this test."

NCI noted that control and dose groups were from different shipments and housed in separate rooms during the studies, and stated:

> "Because of these factors, this increased incidence [of lung tumors in female mice] does not provide sufficient evidence of a compound-related effect. No significant increase in tumor incidence was observed among dosed male mice. Under the conditions of this bioassay, sufficient evidence was not provided to conclusively demonstrate the carcinogenicity of 2,5-toluenediamine [2,5-DAT] sulfate in either Fischer 344 rats or B6C3F1 mice."

The Clearinghouse on Environmental Carcinogens (Clearinghouse), which was established to advise the Director of NCI on its bioassay program, peer reviewed the NCI (1978) studies on 2,5-DAT sulfate (CEC, 1978). The Clearinghouse stated:

"Although a carcinogenic response was not demonstrated, the reviewer said that the evidence was suggestive that the compound may have a carcinogenic potential. He recommended that it be considered for retest. In his critique, he noted several experimental flaws, including the use of animals from different shipments, the conduct of the subchronic study in a different mouse strain than used in the chronic phase, and the start of the high-dose rats on test some months after the initiation of the low-dose animal group. The reviewer said the compound warranted further testing because of the experimental design and study conduct deficiencies, as well as the fact that 2,5-Toluenediamine [2,5-DAT] Sulfate had been shown to be positive in the Ames assay. The reviewer moved that the report on the bioassay of 2,5-Toluenediamine [2,5-DAT] Sulfate be accepted as written but that the compound be considered for retest. The motion was approved without objection."

3.2.3 2,6-DAT

OEHHA identified one NCI (1980) report on the carcinogenicity of 2,6-DAT dihydrochloride in animals (NCI, 1980). The NCI report included four dietary bioassays, one each in male and female F344 rats, and one each in male and female B6C3F1 mice. The dihydrochloride salt is more stable than 2,6-DAT in feed.

3.2.3.1 Long-term 2,6-DAT dihydrochloride feeding studies in male and female Fischer 344 rats (NCI, 1980)

NCI conducted two carcinogenicity studies of 2,6-DAT dihydrochloride in male and female F344 rats (NCI, 1980). In each study, groups of 50 rats were fed diets containing 2,6-DAT dihydrochloride (>99 percent purity) at concentrations of 0, 250, or 500 ppm in the feed. Animals were treated for 103 weeks and observed for an additional week. Animals were six to seven weeks old at the beginning of the studies.

Male

In the study in male rats, survival in treated groups was greater than survival in the controls. At the end of two years, the survival rates were 25/50 (50 percent) in the control group, 34/50 (68 percent) in the low-dose group, and 33/50 (66 percent) in the high-dose group. Although the terminal survival rates were low (especially in the control group), 37/50 (74 percent) animals in the control group, 47/50 (94 percent) in the low-dose group, and 40/50 (80 percent) in the high-dose group survived for more than 100 weeks. NCI concluded that there were a sufficient number of male rats at risk for the

development of late-appearing tumors. At 104 weeks, body weight gain depression was less than 10 percent for the dosed animals, compared to the controls.

Statistically significant dose-dependent trends in the incidences of hepatocellular neoplastic nodule (adenoma) and combined hepatocellular adenoma and carcinoma were observed in 2,6-DAT dihydrochloride treated males (Table 5). Liver hepatocellular carcinomas are rare tumors in male F344 rats. The historical control incidence of spontaneous hepatocellular adenomas or carcinomas at this laboratory is 2/334 (0.6 percent) as reported by NCI (1980). The incidence in National Toxicology Program (NTP) feeding studies conducted up to 1997 is 0.7 percent (range: 0 to 6 percent) (Haseman *et al.*, 1998), and the incidence in NCI Carcinogenesis Testing Program studies conducted between 1972 and 1978 is 24/1794 (1.3 percent) and 7/1794 (0.4 percent) for adenomas and carcinomas, respectively (Goodman *et al*., 1979). Thus, the incidence of combined hepatocellular adenomas or carcinomas in the high-dose group, 9.8 percent, is higher than that of the laboratory historical controls, and higher than that reported for either NTP feeding studies or NCI feeding studies.

A statistically significant dose-dependent increase in pancreatic islet-cell adenoma was also observed in treated males with an incidence in the high-dose group of 9.3 percent (Table 5). Pancreatic islet-cell adenomas are benign tumors with a spontaneous incidence of 4.0 percent (range: 0 to 10 percent) in male F344 rats in NTP feeding studies conducted up to 1997 (Haseman *et al.*, 1998), and 3.5 percent in NCI Carcinogenesis Testing Program studies conducted between 1972 and 1978, as reported by Goodman *et al.* (1979).

Table 5. Tumor incidence[1] in male F344 rats fed diets containing 2,6-DAT dihydrochloride for two years (NCI, 1980)

Tumor Site and Type		Concentration (ppm)			Trend test[2]
		0	250	500	
Liver	Hepatocellular adenoma	0/33	0/42	3/38 (7.9%)	0.036
	Hepatocellular carcinoma (rare)	0/39	2/47 (4.3%)	1/41 (2.4%)	NS
	Combined	0/39	2/47 (4.3%)	4/41 (9.8%)	0.035
Pancreas	Islet-cell adenoma	0/39	1/47 (2.1%)	4/43 (9.3%)	0.025

[1] Tumor incidences are given as the ratio of the number of tumor-bearing animals at a specific anatomic site (numerator) to the number of animals that are still alive at the time of first occurrence of this tumor (denominator).
[2] p-value from exact trend test conducted by OEHHA. NS, not significant

Non-neoplastic findings

An increase of "basophilic cytoplasmic change" was observed in the livers of treated male rats, with incidence of 3/50, 5/50, and 10/50 in the control, low-dose, and high-dose groups, respectively (NCI, 1980). These cytoplasmic changes are recognized as altered hepatocellular foci in hematoxylin and eosin (H&E) stained liver tissue. Altered foci of hepatocytes are commonly seen in rats six months or older in two-year studies (Eustis *et al.*, 1990). There are five main categories: basophilic, eosinophilic, clear, vacuolated, and mixed cell foci. In male rats, eosinophilic and clear cell foci are generally the most common categories. Basophilic foci of hepatocytes may occur randomly or appear as a precursor to neoplasms (Eustis *et al.*, 1990).

Female

In the study in female rats, no treatment-related differences in survival were observed. At the end of two years, survival rates were 35/50 (70 percent) in the control group, 35/50 (70 percent) in the low-dose group, and 39/50 (78 percent) in the high-dose group. NCI concluded that there were a sufficient number of animals at risk for the development of late-appearing tumors. At 104 weeks, the mean body weights of the low-dose and high-dose females were 17 percent and 27 percent lower than controls, respectively.

No treatment-related increases in the incidence of tumors or non-neoplastic findings were reported in this study.

3.2.3.2 Long-term 2,6-DAT dihydrochloride feeding studies in male and female B6C3F1 mice (NCI, 1980)

NCI (1980) conducted two carcinogenicity studies of 2,6-DAT dihydrochloride in male and female B6C3F_1 mice. In each study groups of 50 animals were fed diets containing 2,6-DAT dihydrochloride (>99 percent purity) at concentrations of 0, 50, or 100 ppm in the feed. Both male and female mice were treated for 103 weeks and observed for an additional week.

Male

In the male study, no treatment-related differences were observed in survival or body weight. Survival at the end of the study was 31/50 (62 percent) in the control group, 31/50 (62 percent) in the low-dose group, and 32/50 (64 percent) in the high-dose group. NCI concluded that there were a sufficient number of mice at risk for the development of late-appearing tumors. NCI further noted that the lack of significant weight gain depression or other observable clinical signs of toxicity in the male mice suggested that the maximum tolerated dose may not have been tested.

No treatment-related tumors were observed in the male mice.

Female

In the female study, no treatment-related differences were observed in survival or body weight. Survival at the end of the study was 39/50 (78 percent) in the control group, 40/50 (80 percent) in the low-dose group, and 39/50 (78 percent) in the high-dose group. NCI concluded that there were a sufficient number of mice at risk for the development of late-appearing tumors, and noted that the lack of significant weight gain depression or other observable clinical signs of toxicity suggested that the maximum tolerated dose may not have been tested.

As shown in Table 6, three hepatocellular carcinomas were observed in high-dose females, with none observed in the control or low-dose groups. Although the increased incidence of hepatocellular carcinoma was not statistically significant by pairwise comparison with controls, it was significant ($p<0.05$) by the exact trend test. However, the incidence of hepatocellular adenoma was unaffected by 2,6-DAT dihydrochloride-treatment, and the increase in combined hepatocellular adenoma and carcinoma observed in the high-dose group did not reach statistical significant by pairwise comparison or trend tests. It is notable that the first hepatocellular carcinoma was

observed in week 93 of the study, while hepatocellular adenoma was observed later, at study termination (week 105).

No treatment related non-neoplastic findings were reported in the livers of female mice in this study.

Table 6. Tumor incidence[1] in female B6C3F1 mice fed diets containing 2,6-DAT dihydrochloride for two years (NCI, 1980)

Tumor Site and Type		Concentration (ppm)			Trend test[2]
		0	50	100	
Liver	Hepatocellular adenoma	4/43 (9.3%)	3/44 (6.8%)	4/42 (9.5%)	NS
	Hepatocellular carcinoma	0/46	0/48	3/45 (6.7%)	0.032
	Combined	4/46 (8.7%)	3/48 (6.3%)	7/45 (15.6%)	NS

[1] Tumor incidences are given as the ratio of the number of tumor-bearing animals at a specific anatomic site (numerator) to the number of animals that are still alive at the time of first occurrence of this tumor (denominator).
[2] p-value from exact trend test, conducted by OEHHA. NS, not significant.

3.2.3.3 Conclusions in and peer-review comments on the 1980 NCI 2,6-DAT dihydrochloride studies in male and female F344 rats and B6C3F1 mice

NCI (1980) determined that 2,6-DAT dihydrochloride was not carcinogenic in male F344 rats because both the incidences of hepatocellular tumors and pancreatic islet-cell adenoma were not statistically significantly increased from the controls by Fisher exact pairwise comparison.

Similarly, NCI (1980) determined that 2,6-DAT dihydrochloride was not carcinogenic in female B6C3F1 mice based on the non-significant increase in tumor incidence between treatment groups and controls, by pairwise comparison.

The Clearinghouse on Environmental Carcinogens (Clearinghouse) peer reviewed the NCI (1980) studies on 2,6-DAT dihydrochloride (CEC, 1980). The Clearinghouse stated:

> The primary reviewer for the report on the bioassay indicated, “a dose-related incidence of liver tumors occurred in treated male rats and female mice. He opined that the maximum tolerated dose was not reached. The reviewer pointed out that the mice receiving 2,4-TDA [2,4-DAT] were given a dose about two times

the one used in this study. Based on these findings and certain inconsistencies in the language of the write-up, he recommended that the report on the bioassay of 2,6-TDA [2,6-DAT] not be accepted. He said that acceptance of the report would indicate that the study was adequate and the chemical need not be further tested."

"The secondary reviewer agreed with some of the reservations expressed by the primary reviewer. Despite the reservations, he said the conclusion that the chemical was not carcinogenic, under the conditions of test, was valid."

"A Clearinghouse member suggested that a statement be added to the report indicating the need for further testing to resolve questions raised by deficiencies in this study. Another member agreed that such a statement should be included, especially since there is a wide variety of species differences to aromatic amines. He suggested that the hamster might be an appropriate test species. "

3.2.4 Complex Mixtures Containing One or More DAT Isomers

Six rodent carcinogenicity studies were identified of complex mixtures containing one or more DAT isomers. Each of these studies are dermal application studies. Three studies were conducted in rats and tested complex mixtures containing 2,5-DAT. Three studies were conducted in mice and tested complex mixtures containing 2,5-DAT and 2,4-DAT. For a number of reasons, including the relatively low levels of 2,5- and 2,4-DAT present in the tested mixtures, and the presence of multiple compounds in these mixtures, these studies have limited usefulness for the cancer hazard identification of DATs. For the sake of completeness, these studies are briefly discussed below.

3.2.4.1 Dermal studies of complex mixtures containing 2,5-DAT in rats

Two-year skin painting studies in male and female Sprague Dawley rats (Kinkel and Holzmann, 1973)

Sprague Dawley (SD) rats (males and females) were treated with a carboxymethyl cellulose gel containing either four percent 2,5-DAT, three percent 2,5-DAT along with 0.75 percent 2,4-diaminoanisole, or gel without dye added. Each formulation was mixed with an equal volume of six percent hydrogen peroxide immediately before use, and 0.5 g of the mixture was applied to the dorsal skin of male and female rats. Rats were treated twice weekly for two years with either formulation or the control gel, and one control group received no treatment at all. The study did not report pathology data

separately for male and female rats. Tumor incidence and survival were comparable in all experimental and control groups, and no tumors developed at the site of application. IARC (1978) noted the absence of information about survival times and the high incidence of tumors in the controls.

Two generation reproduction and chronic toxicity studies in male and female SD rats (Burnett and Goldenthal, 1988)

Oxidative hair coloring formulations containing 3 or 6 percent 2,5-DAT respectively were applied to the shaved backs of male and female SD rats. The dyes were mixed with an equal volume of 6 percent hydrogen peroxide and the mixtures contained other hair dye chemicals. 0.5 mL of hair dye was applied per rat until the animals reached 100 days of age. The treated rats were then paired and mated, and 60 male and 60 female pups of the F1 generation were selected for the chronic toxicity/carcinogenicity studies. In each study, pups were treated with the same hair dye formulations for approximately two years, and the studies were terminated at 117 weeks. There were no differences in body weight between the treated and untreated groups in either study. The incidence of neoplasms was highly variable in all groups and could not be correlated to hair dye exposure in either study.

3.2.4.2 Dermal studies of complex mixtures containing 2,5-DAT or 2,5-DAT sulfate and 2,4-DAT in mice

18-month skin painting studies in male and female Swiss Wester mice (Burnett *et al.*, 1975)

Male and female random-bred Swiss Webster mice were treated with 0.5 mL of a hair dye formula containing 3 percent 2,5-DAT sulfate applied once weekly or every other week to the shaved mid-scapular area for an 18-month period. In each study, there were 50 mice in each treatment group (including the vehicle control group), and 125 mice in the untreated control group. The hair dye was mixed with an equal volume of 6 percent hydrogen peroxide just prior to use. Three different hair dye formulas were tested, each containing 3 percent 2,5-DAT sulfate, with either phenylenediamine and 2,4-DAT, or 2,4-diaminoanisole sulphate. The vehicle control group received the base hair dye formula with no added dye. A positive control group received 7,12-dimethylbenz[*a*]anthracene in acetone. Survival at 18 months in each study ranged from 62 to 66 percent in the treated groups compared to 78 percent in the untreated controls and 21 percent in the positive controls. No treatment-related tumors were observed in either study.

Two-year skin painting study in male and female (combined) Swiss Webster mice (Giles *et al.*, 1976)

Two different formulations of oxidative hair dye containing 3 percent 2,5-DAT, mixed with an equal volume of 6 percent hydrogen peroxide, were applied to the shaved interscapular region of male and female Swiss Webster mice. There were 28 male and 28 female mice in each treatment group, 14 male and 14 female mice in the acetone group, and 76 male and 17 female mice in the untreated control group. Mice received application of 0.05 mL final hair dye solution weekly for two years. The hair dye formulations also contained *p*-phenylenediamine, resorcinol, and 2,4-DAT. No differences in weight gain were noted between treated and untreated groups, and no treatment-related increases in tumors were observed.

3.3 Other Relevant Data

3.3.1 Pharmacokinetics and Metabolism

The pharmacokinetics and metabolism of DATs have been studied with select isomers, i.e., 2,4-DAT, 2,5-DAT and 2,6-DAT, and their salts; no studies of the other isomers were identified. In addition to the peer-reviewed journal articles found in the literature, OEHHA identified a review conducted by the Scientific Committee on Consumer Safety (SCCS) of the European Commission abstracting a number of unpublished studies on the absorption, distribution, metabolism, and elimination of 2,5-DAT and its sulfate. The SCCS (2012) review included limited information on study design and results from these unpublished studies. Information on these unpublished studies, as reported in SCCS (2012), is included here.

Limited human *in vivo* studies on the disposition of 2,4- and 2,5-DAT are available, primarily via the dermal route, with one study using subcutaneous (*s.c.*) injection. Several human studies also examined the disposition of the 2,5-DAT isomer contained in commercial or specifically formulated hair dye mixtures. A few studies evaluated *in vitro* absorption from donated human skin and *in vitro* metabolism from donor or cadaver liver preparations. Additionally, a study on monkeys measuring dermal absorption of 2,4-DAT was identitied in the literature.

Pharmacokinetic and metabolism studies in non-primates were conducted *in vivo* on mice, rats, dogs, hamsters, guinea-pigs, and rabbits, via various routes of administration: oral gavage, dermal, intraperitoneal (*i.p.*) injection, intravenous (*i.v.*)

infusion, and *s.c.* injection. There were three strains of rats (Fischer, Kyoto, and SD) and two strains of mice (Swiss and B6C3F[1][10]) used in the *in vivo* studies. Absorption studies were also conducted *in vitro* using a pig skin infusion model and metabolism studies were conducted using cytosolic preparations from mice, rats, hamsters, rabbits, guinea-pigs and one dog. 2,5-DAT has been extensively studied, but studies on 2,4- and 2,6-DAT were limited and no studies were available for the other three DAT isomers.

3.3.1.1 Pharmacokinetic studies - humans and monkeys

Absorption

Absorption of DATs in humans has not been well studied. OEHHA identified six dermal absorption studies in the literature: one on 2,4-DAT and five on 2,5-DAT sulfate (four in commercial hair dye formulations). No studies on absorption through other routes were identified. These dermal studies are briefly discussed below.

Marzulli *et al.* (1981) conducted a dermal *in vivo* absorption and penetration study on five chemicals, including 2,4-DAT [2,4-TDA]. Radiolabeled ^{14}C-2,4-DAT dihydrochloride [ring-labeled, 5.28 mCi, specific activity 2.78 mCi/mmole] was dissolved in acetone and applied to groups of three to six consented humans (adult males), and rhesus monkeys (adult males and females). Radiolabeled 2,4-DAT dihydrochloride solution was painted on the ventral surface of the forearm in the humans and on the abdomen in the monkeys at a dose of 4 µg/cm^2 over a skin area of 3 to 15 cm^2. At 24 hours after the application, the application sites were washed with soap and water. Total radioactivity in the urine sampled over a five-day period was assumed to be the total amount of 2,4-DAT dermally absorbed in the first 24 hours of application. Based on radioactivity levels in the urine over a five-day period, the authors calculated a 23.7 percent and 53.8 percent dermal absorption over the 24-hour period, in humans and monkeys, respectively. The data suggest that compared to monkey skin, human skin may be less permeable to 2,4-DAT (Marzulli *et al.*, 1981).

A second dermal absorption study in adult humans measured the uptake of 2,5-DAT applied in a laboratory formulated hair dye (Kiese and Rauscher, 1968). 2,5-DAT sulfate (2.5 grams) was mixed with an equal amount of resorcinol and added to a solution containing 50 mL of water and 50 mL of a fluid consisting of oleic acid, isopropanol, ammonia, higher alcohol sulfates, sodium ethylenediamine tetraacetate,

[10]Unger *et al.* (1980) reports that B6C3F mice were used in the study. The mouse strain is a hybrid of B6 and C3 mouse strains, with the first generation offspring usually referred to as B6C3F1 mice.

sodium sulfate, and perfume. The formula was applied to the hair and scalp of four females and one male for 40 minutes. The hair and scalp were then washed and dressed. Urine samples were collected and N,N'-diacetyl-*p*-toluenediamine (a 2,5-DAT metabolite) levels were measured at various time points within 48 hours after the application of hair dye. The chemical was detected in the urine within the first four hours and the highest rate of urinary excretion occurred between five to eight hours after application, indicating rapid absorption. The study authors calculated an average amount of 4.6 mg equivalent of 2,5-DAT was absorbed during the 40 minutes of hair dye application, which was less than 0.2 percent of the applied dose (Kiese and Rauscher, 1968).

Schettgen (2011) quantified the amount of chemicals (including 2,5-DAT) taken up during personal use of commercial hair dye. 2,5-DAT (amount unknown) was applied as part of a commercial hair dye by two adult females. Each female wore gloves and applied a different shade of hair dye onto her hair. Thirty minutes after the application, the hair dye was removed by washing. Urine samples were collected over a 48-hour period and analyzed for 2,5-DAT. The highest urinary 2,5-DAT levels were reached at 2.5 and 4.2 hours, suggesting a rapid dermal absorption (Schettgen, 2011).

In a review on 2,5-DAT and its sulfate, the SCCS describes two dermal absorption studies on 2,5-DAT sulfate in hair dye formulations (SCCS, 2012). The first study examined uptake following dermal 30-minute application of two hair dye formulations (non-oxidative or oxidative), each applied to the scalp of five adult males. Forty-five grams of hair dye formulation containing ^{14}C-2,5-DAT sulfate [0.825 percent, ^{14}C-CH_3 labeled, purity unspecified] in the presence or absence of six percent hydrogen peroxide (oxidative or non-oxidative formulation) was applied to the scalp, for a total dose of 1.48 mg/cm^2 ^{14}C-2,5-DAT sulfate. After 30 minutes, the hair dye was rinsed off and the hair was shampooed. Based on radioactivity levels in the urine and feces collected over a 48-hour period, the authors estimated dermal absorption of 4.81 percent and 1.31 percent of the applied dose for the non-oxidative and oxidative formulations, respectively. The overall recoveries of radiolabel were reported as 86 and 47 percent for the non-oxidative and oxidative formulations, respectively (Aylward, 1981, as reported in SCCS, 2012, pp. 53-54). Additional information on radioactivity levels measured in the blood or urine at earlier time points was not provided in the SCCS report (SCCS, 2012).

In the second study, a mass balance study (Cosmetic Ingredient Review Expert Panel, 1992), two oxidative hair dye formulations containing different amounts of 2,5-DAT sulfate (“low” and “high” DAT formulations) and ^{14}C ring-labeled 2,5-DAT sulfate [80 mCi/mmol, 97.8 percent purity] were applied to the hair of two groups of 16 adults (27

males and 5 females). The final on-head concentrations were 1.5 and 4 percent DAT, plus couplers, following mixing with hydrogen peroxide-containing developer (1:1, w/w). The dye was rinsed off and the hair was shampooed, dried and clipped after 30 minutes. The scalp was then capped overnight and washed the following morning. Urine, feces, and blood samples were collected over a 48-hour period. Overall recoveries (washing water, clipped hair, protective caps, and other materials) were 99 and 98 percent for the high and low DAT hair dye formulations, respectively. The authors determined that the average dermal absorption was approximately 0.9 percent of the administered dose for both hair dye formations, based on the amount of radioactivity in the urine and feces collected over the 48-hour period. This study was briefly reported by the SCCS and no data describing the radioactivity-time profile in the blood or urine were available.

The SCCS (2012) reported an *in vitro* percutaneous absorption study with 2,5-DAT sulfate (TNO, 2009, as reported in SCCS, 2012, pp. 22-25). Eighteen human skin samples from the abdomen, breast, or back were obtained from three donors. Dermal absorption of 2,5-DAT sulfate was measured using an automatic Teflon flow-through chamber. Radiolabeled ^{14}C- 2,5-DAT sulfate [>99 percent purity, 72 mCi/mmol] and non-labeled 2,5-DAT sulfate was added to hair dye formulations (i.e., color cream) to obtain a series of 2,5-DAT concentrations. Hair dye formulations containing 0.25, 0.8, 2.4, or 7.2 percent 2,5-DAT (with or without hydrogen peroxide) were applied to human skin in the flow chamber at a dose of 20 mg/cm^2 of the final formulation over a skin area of 0.64 cm^2. The prepared skin samples were washed 30 minutes after the hair dye application. Total dermal absorption was calculated from the total radioactivity levels in the receptor fluid, the receptor compartment wash collected over a 24-hour period, and the skin. Total absorption of the radiolabel ranged from 3.65 to 9.55 percent and 3.49 to 3.90 percent for non-oxidative (without hydrogen peroxide) and oxidative (with hydrogen peroxide) conditions, respectively. In general, the total absorption increased with increasing amounts of 2,5-DAT. Similar to the results in Aylward (1981, as reported in SCCS, 2012), the presence of hydrogen peroxide, which is commonly used in oxidative hair dyes, reduces the total amount of DAT absorbed (Leclerc, 1997, and TNO, 2009, both as reported in SCCS, 2012, pp. 22-26).

Distribution

Distribution of 2,5-DAT following topical applications of hair dye was examined in three studies.

In the study by Schettgen (2011), a commercial hair dye containing unknown amounts of 2,5-DAT was applied to the hair of two adult females. Urinary level of 2,5-DAT

peaked at 2.5 to 4 hours, suggesting a rapid distribution following dermal absorption (Schettgen, 2011).

Aylward (1981) (as reported in SCCS, 2012) examined the distribution of 2,5-DAT in the blood following dermal application of ^{14}C-labeled 2,5-DAT sulfate containing-hair dye to the scalp of five male adults. The half-life of radioactivity in the blood was between one to three hours.

In a mass balance study reported by the Cosmetic Ingredient Review Expert Panel (1992), radioactivity levels peaked in the blood, with the time to reach peak concentration in blood (T_{max}) at 2.4 and 2.6 hours and a half-life of 10.1 and 11.3 hours for the low- and high-dose groups, respectively. The SCCS stated that there was a four-fold difference in the area under the curve values of radioactivity in the plasma in both dose groups. The SCCS concluded, "The variability observed in the individual results is likely due to the variation in plasma levels that is frequently observed in studies performed via topical application."

Metabolism

OEHHA identified one *in vitro* study on metabolism of 2,4-DAT, one *in vivo* study and two *in vitro* studies on metabolism of 2,5-DAT (one used the sulfate salt).

An *in vitro* metabolism study on 2,4-DAT is available in the literature (Glinsukon *et al.*, 1975). Incubation of 2,4-DAT [minimum 99 percent purity] with liver cytosol, isolated from autopsy tissues of three men, yielded trace levels of 4-acetylamino-2-aminotoluene. The authors concluded that delays caused by autopsy may not be the cause of low N-acetylation activity in the human cytosol, since cytosolic preparations obtained from frozen livers of rabbit (frozen over one month) or hamster (frozen for one week) retained over 50 and 75 percent of the N-acetylation activity, respectively. The authors further concluded that humans have a low capability to N-acetylate 2,4-DAT. However, information on the stability of enzyme activity in stored and frozen liver cytosol preparations derived from animal tissues does not directly address the concerns regarding enzyme activity in liver cytosol preparations derived from human autopsy material. This is because there is generally a longer lag time, often on the order of hours, between death and the removal and processing of human autopsy material, as compared to the much shorter lag time, typically on the order of minutes, for obtaining and processing animal tissues. In addition, the temperature conditions at which the human autopsy material is kept, prior to removal and processing, will also affect the stability of the enzymatic activity.

An *in vivo* metabolism study on 2,5-DAT is available in the literature (Kiese and Rauscher, 1968). N,N'-Diacetyl-*p*-toluenediamine, an acetylation metabolite of 2,5-DAT, was formed and was detected in the urine after application of 2,5-DAT sulfate to the scalp, or *s.c.* injection of 2,5-DAT to four adult females and one adult male. The authors calculated that 48 percent of the injected dose was metabolized to N,N'-diacetyl-*p*-toluenediamine and excreted in the urine.

Two *in vitro* metabolism studies are available on 2,5-DAT (one used the sulfate salt). In the first *in vitro* metabolism study, Krebsfanger (2003, as reported in SCCS, 2012, p. 49) used cryopreserved primary human hepatocytes harvested from three male donors (rapid N-acetylation phenotype) to investigate the metabolism of 2,5-DAT in humans. 2,5-DAT sulfate [98.3 percent purity] was completely metabolized after four hours of incubation in the cultured hepatocytes, with only one mono-N-acetylated metabolite detected (structure not specified). The study authors stated that 2,5-DAT is a substrate for both N-acetyltransferase 1 (NAT1) and NAT2. The SCCS commented that no conclusion could be drawn from this study regarding the relative activities of these N-acetylation isozymes in the metabolism of 2,5-DAT.

Another *in vitro* study was conducted by Skare *et al.* (2009) (also reported by Powrie, 2005, as reviewed in SCCS, 2012, p. 50) using isolated human hepatocytes (pooled from four female donors), liver microsomal preparations, or human recombinant CYP isozymes (rCYP1A1, rCYP1A2, rCYP1B1, rCYP2C9, rCYP2C19, rCYP2D6, or rCYP3A4). Incubations of 10 or 100 µM ^{14}C radiolabeled 2,5-DAT [specific activity 55 mCi/mmol] with human hepatocytes generated a mono-acetyl metabolite (5-acetyl-2,5-DAT) in the first 60 minutes and a trace level (one to two percent) of a di-acetyl metabolite detected after 120 to 240 minutes of incubation. No oxidative or other conjugated metabolites were detected. Approximately 10 percent of the 2,5-DAT was metabolized by the hepatocytes over an incubation period of 240 minutes. Incubations of human microsomes with 2,5-DAT yielded no oxidized metabolites (hydroxy or di-oxygenated metabolites), whereas 2-aminofluorene (2-AF, a positive control) was mono-hydroxylated at multiple positions. Similarly, incubation of rCYPs with 2,5-DAT produced no hydroxylated metabolites, whereas incubations of rCYPs with 2-AF produced 1-hydroxy, 3-hydroxy, 5-hydroxy, and N-hydroxy-metabolites. The study authors concluded that 2,5-DAT is metabolized via N-acetylation in human hepatocytes, and that no CYP-mediated metabolism or glucuronide conjugation occurs in human hepatocytes or microsomes or with human recombinant CYP isozymes.

Elimination

Limited studies are available on the elimination of DAT isomers and their metabolites in humans, and only one study was conducted in monkeys. These consist of one study in humans and monkeys on 2,4-DAT following dermal application (Marzulli *et al.*, 1981), four studies in humans on 2,5-DAT (and its sulfate) following dermal application of hair dye formulations (Kiese and Rauscher, 1968; Aylward, 1981; Cosmetic Ingredient Review Expert Panel, 1992; Schettgen, 2011) (the Aylward study was reported in SCCS, 2012), and one study in humans on 2,5-DAT following subcutaneous injection (Kiese and Rauscher, 1968). Most of these studies measured urinary excretion of ^{14}C after administration of the labeled compound. Only one study measured a metabolite of 2,5-DAT in the urine (Kiese and Rauscher, 1968).

Marzulli *et al.* (1981) examined the urinary excretion of radioactivity following a single dermal application of radiolabeled ^{14}C-2,4-DAT to groups of three to six adult men and adult male and female rhesus monkeys. Over a four-day period, urine radioactivity levels peaked at 12 to 24 hours and 8 to 12 hours in humans and monkeys, respectively. While urine radioactivity levels in humans dropped to negligible levels (less than one percent of absorbed dose) in days two and three, radioactivity levels in the urine of monkeys were 3.7 and 1.8 percent of the absorbed dose for the second and third days, respectively.

Schettgen (2011) measured urinary excretion of 2,5-DAT over a 48-hour period in two female adults (slow acetylators), following dermal application of 2,5-DAT containing hair dye. Urinary elimination of 2,5-DAT followed first-order kinetics, with a half-life of 8 to 10 hours. Although the urinary 2,5-DAT levels peaked rapidly at 2.5 and 4 hours (for the two individuals, respectively) and most of the 2,5-DAT was excreted in the urine within the first 24 hours (90 percent), 2,5-DAT was still detectable in the urine at 48 hours following application (Schettgen, 2011).

In the hair dye absorption study conducted by Kiese and Rauscher (1968), urinary 2,5-diacetylaminotoluene [N,N'-diacetyl-*p*-toluenediamine] (a 2,5-DAT metabolite) levels were monitored for 48 hours after the application of 2,5-DAT sulfate containing hair dye. The highest rate of 2,5-diacetylaminotoluene excretion in the urine occurred between five to eight hours after application and trace levels of the metabolite were found at 36 hours. The authors estimated that 2.17 mg of the administered 2,5-DAT dose was excreted (based on 3.66 mg 2,5-diacetylaminotoluene excreted), corresponding to less than 0.1 percent of the original applied dose.

Additionally, Kiese and Rauscher (1968) examined the urinary excretion of 2,5-diacetylaminotoluene [N,N'-diacetyl-*p*-toluenediamine] following *s.c.* injection of 5.54 mg 2,5-DAT equivalent (the sulfate salt was used in the injection) to three male and three female adults. Most of the urinary excretion of 2,5-diacetylaminotoluene occurred in the first two days following the injection, with an average of 48 percent of the administered dose found in the urine over the three-day period.

In the hair dye absorption study conducted by Aylward (1981, as reported in SCCS, 2012), dermal application of hair dye containing ^{14}C radiolabeled 2,5-DAT sulfate, with or without hydrogen peroxide, to five male adults resulted in 2.3 and 0.8 percent of the applied dose excreted in the urine over a 48-hour period, under non-oxidative and oxidative conditions, respectively. The combined excretion in the urine and feces was 4.81 percent under non-oxidative conditions and 1.31 percent under oxidative conditions (Aylward, 1981, as reported in SCCS, 2012).

In a similar study, ^{14}C-radiolabeled 2,5-DAT was applied at two concentrations (1.5 or 4 percent 2,5-DAT) in a hair dye to two groups of 16 male and female adults. The mean urinary excretion for either concentration was 0.83 percent of the applied dose over a 48-hour period. Within the same time period, excretion in the feces was 0.035 to 0.04 percent of the applied dose for both the low- and high-dose groups. The overall excretion was 0.9 percent of the applied dose over the 48-hour period in both dose groups (Cosmetic Ingredient Review Expert Panel, 1992).

3.3.1.2 Pharmacokinetic studies - non-primates

OEHHA identified the following *in vivo* metabolism studies:

- 2,4 DAT- one oral study in rats and four *i.p.* studies in rats and mice
- 2,5 DAT- five dermal studies in rats and dogs, four oral studies in rats, two *i.v.* studies in rats, one i.v. study in dogs, one *s.c.* study in rats
- 2,6 DAT- one oral study in rats

Furthermore, the following *in vitro* studies were identified:

- 2,4-DAT- two studies using rat tissue, two studies using hamster tissue, one study each using rabbit, guinea pig or mouse tissue
- 2,5-DAT- one rat study, one mouse study, and two studies using the pig skin perfusion model

Absorption

Oral

2,4-DAT

Timchalk *et al.* (1994) administrated a single dose of 3 or 60 mg/kg ^{14}C-radiolabeled 2,4-DAT to male F344 rats via oral gavage. A separate group of male F344 rats (number unspecified) were treated with 3 mg/kg radiolabeled 2,4-DAT via *i.v.* infusion. Urine was collected over a 48-hour period post-treatment. For all the treatment groups, radioactivity recovery was between 93 and 97 percent of the administered doses. The urinary radioactivity recoveries were similar between the 3 mg/kg oral and *i.v.* infusion treatments, 64 verses 72 percent, respectively. Oral absorption was approximately 70 percent of the administered dose, estimated from the total radioactivity in the urine over a 48-hour period, and in tissue, and the carcass. Together, these data suggested that 2,4-DAT was well absorbed following oral administration and that the oral absorption of 2,4-DAT was not dose-dependent in this study.

2,5-DAT

Hruby (1977) administered one mL of a 1.6 percent ^{14}C-labeled 2,5-DAT hydrochloride [specific activity 15.8 mCi/g 2,5-DAT, purity unknown] aqueous solution via oral gavage to a group of nine male and nine female SD rats. Urine and feces were collected daily for five days, at which point the animals were killed, the gastro-intestinal (GI) tract removed, and the rest of the body homogenized. Following oral gavage, a large fraction of radioactivity (slightly above 70 percent of the applied dose) showed up in the urine

within the first day. 2,5-DAT was extensively absorbed in the GI tract following oral administration in the rats.

Wenker (2005c) (as reported in SCCS, 2012, p. 52) investigated the oral absorption of 2,5-DAT sulfate [ring ^{14}C-radiolabeled, 99.3 percent purity] in six female SD rats. Rats were dosed via oral gavage with 2.5 or 25 mg/kg bw 2,5-DAT. A rapid oral absorption was evidenced by blood radioactivity level peaking at one hour following oral gavage.

Charles River (2010) (as reported in SCCS, 2012, pp. 56-57) also examined the oral absorption of 2,5-DAT. Groups of six male and six female fasted SD rats were treated with a single oral gavage dose of 5 or 10 mg/kg 2,5-DAT sulfate [ring-labeled ^{14}C-radiolabeled, purity 99.5 percent]. The time to maximum concentration (T_{max}) in the blood was reached at approximately 0.5 to 1 hour after oral dosing.

Dermal (*in vivo*)

2,5-DAT

In one study, six female Kyoto rats (an animal model for slow acetylation) were treated for 30 minutes with a dermal application of 33.3 mg/kg bw 2,5-DAT sulfate [radiolabeled uniformally ring-labeled (U)-^{14}C] in water and acetone. The average dermal absorption was 16 percent of the applied dose. Four percent of the applied dose was detected in the skin 96 hours after application (Wenker, 2005b, as reported in SCCS, 2012, pp. 50-51).

In another study, groups of four female SD rats received 1.4 (21 µg/cm^2), 6 (75 µg/cm^2), 19.5 (224 µg/cm^2), 63.2 (734 µg/cm^2), 1.5 (80 µg/cm^2), or 13.3 (677 µg/cm^2) mg/kg 2,5-DAT sulfate [radiolabeled ring-U-^{14}C] via dermal application for 30 minutes on a shaved area on the back. T_{max} in the blood was reached at approximately 0.5 to 1 hour after dermal application, suggesting a relatively rapid dermal absorption in the rats (NOTOX, 2009, as reported in SCCS, 2012, pp. 54-56).

Wenker (2005c) (as reported in SCCS, 2012) conducted two experiments to compare the absorption of 2,5-DAT sulfate [ring ^{14}C-labeled, 99.3 percent purity] in two different vehicles via dermal application. Two groups of six female SD rats were administered 33.3 mg/kg 2,5-DAT sulfate (0.5 mg/cm^2) via dermal application for 30 minutes. The T_{max} in the blood for dermal application was two hours for both groups.

Dermal application of radiolabeled ^{14}C-2,5-DAT [CH_3 labeled] (hydrochloride salt) was administered to rats in a metabolism study by Hruby (1977). Two different hair dye

formulations (0.5 g) were applied to a skin area of 30 x 30 cm^2 at the dorsal region (hair clipped) of nine male and nine female SD rats. Formulation 1 contained 7.5 mg 2,5-DAT [specific activity of 77.8 mCi/g 2,5-DAT], formulation 2 contained 7.5 [m]g[11] 2,5-DAT [specific activity of 22.2 mCi/g 2,5-DAT], and both formulations contained three percent hydrogen peroxide. The two formulations were applied to the skin surface for 30 minutes. The skin was then washed with shampoo and water and dried. The animals were killed after 24 hours, the skin was removed and the carcass was homogenized. Radioactivity was predominantly found in the shampoo water (96 and 90 percent of the applied doses for formulations 1 and 2, respectively) and application area of skin (five and nine percent of the applied doses for formulations 1 and 2, respectively), suggesting a slow absorption of 2.5-DAT via dermal uptake. Based on the total amounts of radioactivity recovered in the urine, feces, and carcass, the authors estimated a dermal absorption of 0.2 percent of the applied dose within the first day for both the formulations.

In the same report of Hruby (1977), a single dermal application [followed three weeks later by *i.v.* infusion] of 2,5-DAT (hydrochloride salt) was administered to a group of three male and three female dogs. The dogs received 50 mL of a hair dye formulation, containing 1.4 g of 2,5-DAT [specific activity of 32 mCi/g 2,5-DAT] on a skin surface area of 20 x 35 cm^2 in the lateral abdominal region. The dyed skin area was shampooed and washed with warm water three hours after the application. Muzzles were used to prevent the dogs from licking the treatment area. Blood, urine, and feces were collected for five days. Three weeks after the dermal application experiment, the dogs received 0.224 g ^{14}C-2,5-DAT hydrochloride [0.14 g 2,5-DAT, specific activity 15.2 mCi/g 2,5-DAT] in water via *i.v.* infusion at a rate of 9 mL/hour for three hours. Blood, urine, and feces were collected over another five-day period. Using the blood radioactivity level curves of dermal and *i.v.* administrations, the authors estimated a dermal absorption of 0.1 percent of the applied dose in the dogs (Hruby, 1977).

In another dog study (Kiese *et al.*, 1968), 1.4 g 2,5-DAT (sulfate salt) in a gel mix (without hydrogen peroxide) was applied to the abdominal skin (20 x 25 cm^2) of male dogs for three hours. The application site was washed with soap and water. A separate group of dogs was treated with 2,5-DAT (sulfate salt) via *i.v.* infusion. Blood and urinary 2,5-DAT concentrations were measured. At three hours post-treatment, 2,5-DAT concentrations in blood reached the same levels for both treatment groups. The authors estimated that about 40 mg of 2,5-DAT was infused during the three-hour

[11] Hruby (1977) notes, "Formulation 2 (0.5 g containing 7.5 g *p*-tolueneiamine [2.5-DAT] with a specific activity of 22.2 mCi/g *p*-toluenediamine)". Based on the 2,5-DAT hydrochloride (1.5 percent) content in formulation 2, a 0.5 g of formulation 2 should contain 7.5 mg 2,5-DAT dihydrochloride or 5.8 mg 2,5-DAT.

i.v. infusion and determined that the dermal absorption was 40 mg 2,5-DAT (three percent) during the three-hour dermal exposure. The authors estimated that less than 3 mg of 2,5-DAT (<0.2 percent) was absorbed under the oxidative condition. The presence of hydrogen peroxide in the hair dye formulation reduced dermal absorption of 2,5-DAT in dogs.

Dermal (*in vitro*)

2,5-DAT

Two *in vitro* dermal absorption studies using a pig skin and perfusion chamber model were reported in the SCCS review (SCCS, 2012). Wyss (2004) applied a hair dye formulation (without hydrogen peroxide or coupler) containing 4.6 percent 2,5-DAT sulfate [^{14}C-radiolabeled, 98.2 percent purity] onto pig skin (100 mg/cm^2), which was mounted in a perfusion chamber. Total dermal absorption was reported to be 0.2 to 0.4 percent of the applied dose at 72 hours following the application (Wyss, 2004, as reported in SCCS, 2012, pp. 27-28).

In the second study, Bornatowicz (2002) conducted a 30-minute dermal absorption measurement using a similar pig skin *in vitro* model with three different 2,5-DAT sulfate solutions. Solution A was a hair dye formulation containing resorcinol coupler and 5.4 percent 2,5-DAT sulfate. Solution B was a hair dye formulation containing coupler, hydrogen peroxide, and 5.4 percent 2,5-DAT sulfate. Solution C was a 5.4 percent 2,5-DAT sulfate aqueous solution. The average total absorption measured was 1.7, 2.4, and 3.9 percent for solutions A, B, and C, respectively (Bornatowicz, 2002, as reported in SCCS, 2012, pp. 26-27).

Distribution

2,4-DAT

^{14}C-Radiolabeled 2,4-DAT hydrochloride was administered by *i.p.* injection to groups of five male B6C3F[1][12] mice at a dose of 0.667 mg/kg. Blood was sampled and organs and tissues were taken at necropsy between 0.5 and 24 hours following the injection (Unger *et al.*, 1980). Radioactivity levels peaked in most of the tissues between half to one hour post injection. Relatively high radioactivity levels were observed in the GI tract, lymph nodes, eyes, and lungs. The radioactivity levels in the large intestine

[12] Unger *et al.* (1980) reports that B6C3F mice were used in the study. The mouse strain is a hybrid of B6 and C3 mouse strains, with the first generation offspring usually referred to as B6C3F1 mice.

increased rapidly within the first hour. There was also a slight increase in radioactivity levels in the large intestine from 16 to 24 hours, when radioactivity levels were decreased in most of the organs. Liver and kidney had higher concentrations of radioactivity compared to blood at all times. The authors associated the high levels of radioactivity in the kidney with elimination of 2,4-DAT in the urine. There were large amounts of radioactivity in the muscle and skin, which might be due to the large relative organ mass for muscle and skin. The radioactivity concentrations in muscle never exceeded the concentrations in the blood.

Grantham *et al.* (1979) conducted metabolism studies in male Fischer rats and male Swiss mice via *i.p.* injection of 77 mg/kg 2,4-DAT. Distributions of 2,4-DAT to the blood and plasma of rats and mice after *i.p.* injection were rapid and peaked within one hour following the injection. Similar distribution patterns were noted in rats and mice: radioactivity detected in the liver, kidneys, lungs, spleen, heart, testes, blood, and plasma within one to 24 hours after the *i.p.* injection. Among the tissues examined, kidney and liver had the highest amounts of radioactivity at both 1 and 24 hours post injection. Overall, mouse tissue had a lower amount of radioactivity compared to rat (Grantham *et al.*, 1979).

In the studies conducted by Timchalk *et al.* (1994), tissue distributions of 2,4-DAT were compared between oral gavage and *i.v.* infusion in rats. At 48 hours post-treatment, the amount of radioactivity recovered was 64 to 75 percent in the urine (collected over 48 hours), 20 to 31 percent in the feces (collected over 48 hours), two to five percent in the tissues and carcass, and less than one percent in the cage wash for oral and *i.v.* infusion, respectively. The minimal amount in the carcass indicated that 2,4-DAT did not accumulate in the body of rats following either *i.v.* infusion or oral ingestion (Timchalk *et al.*, 1994).

2,5-DAT

In a study by Hruby (1977) (also described above) a 0.4 percent aqueous solution of 2,5-DAT hydrochloride (specific activity 12.8 mCi/g 2,5-DAT) was injected subcutaneously (1 mL containing 3 to 5 mg 2,5-DAT) into the dorsal region of another group of nine male and nine female rats. Urine and feces were collected daily for five days, at which point the animals were killed. Following *s.c.* injection a large fraction of radioactivity (slightly less than 70 percent of the applied dose) appeared in the urine within the first day. Approximately seven percent of the applied radioactivity was recovered from the carcass and two percent remained at the application site, five days after the *s.c.*injection. These data suggest that 2,5-DAT and/or its metabolites were

slowly distributed to the circulatory system (residual level in the injection site at day five) without accumulating in the body.

Hruby (1977) also administered a 1.6 percent aequous solution of 2,5-DAT hydrochloride [specific activity of 15.8 mCi/g 2,5-DAT] to nine male and nine female rats via oral gavage. Similar to the *s.c.*treatment, a large fraction of radioactivity (slightly more than 70 percent of the applied dose) appeared in the urine within the first day. At day five, 1.4 percent of administered radioactivity remained in the GI tract, and 1.2 percent was found in the body homogenate.

Additionally, Hruby (1977) compared the distribution of ^{14}C labeled 2,5-DAT following dermal absorption and *i.v.* infusion in dogs. Three weeks following the dermal application of 2,5-DAT (details in Dermal section above), the same group of three male and three female dogs were used in the *i.v.* infusion study. Each animal received 0.224 g of 2,5-DAT hydrochloride [0.14 g ^{14}C-radiolabeled 2,5-DAT, specific activity 15.2 mCi/g 2,5-DAT] in water via *i.v.* infusion at a rate of 9 mL/hour for three hours. Blood, urine, and feces were collected over a five-day period but data were reported for a four-day period. Blood radioactivity levels peaked at six hours post dermal application and two hours post *i.v.* infusion. Blood radioactivity levels slowly decreased from peak levels, and were still elevated four days after dermal application. On the other hand, a rapid decline in blood levels was observed between 6 to 12 hours post *i.v.* infusion, followed by very low blood radioactivity levels measured up through day four post-infusion. These blood radioactivity profiles revealed a slow distribution of 2,5-DAT from the dermal application site to the circulatory system (Hruby, 1977).

2,6-DAT

The distribution of radiolabeled 2,6-DAT after a single oral dose given to rats showed a wide distribution of radioactivity throughout the body. The percent of administered dose for specific tissues and organs was as follows: large intestine (3.6 percent), muscle (1.0 percent), liver (0.6 percent), skin (0.5 percent), blood (0.2 percent), and small intestine (0.1 percent). The concentration of radioactivity in the liver decreased to 0.4 percent by day three and to 0.2 percent by day six; the concentration in blood remained at 0.2 percent over three days and declined to 0.1 percent by day six (Cunningham *et al.*, 1989).

Metabolism

2,4-DAT (in vivo)

In the male F344 rat study by Timchalk *et al.* (1994), animals were treated with 2,4-DAT via oral gavage or *i.v.* infusion. Urine was collected over a 12-hour period following the administration of 2,4-DAT. In the 60 mg/kg orally treated rats, about 62 percent of the material excreted in the urine was either free or acetylated DAT, and the remaining fraction existed as conjugates (Timchalk *et al.*, 1994). The amounts of acetylated or free DAT were comparable between the 3 mg/kg oral and the *i.v.* treatments, 84 and 87 percent of the total urinary material excreted (i.e., metabolites or parent compound), respectively. Mono-acetylated 2,4-DAT, diacetylated 2,4-DAT, and free 2,4-DAT were identified in the urine of all the treated animals (Timchalk *et al.*, 1994).

Metabolism of 2,4-DAT was also examined in male Fischer rats and male Swiss mice by *i.p.* injection of ^{14}C-radiolabeled 2,4-DAT [ring labeled, 2.08 mCi/mmol] (Grantham *et al.*, 1979). The urinary metabolites were separated into four fractions. The 'free metabolite' fractions, which contained 21 and 20 percent (for rats and mice, respectively) of the radioactivity content in the urine, were analyzed to characterize the metabolites. A 4-acetyl derivative and a 2,4-diacetyl compound were the main free metabolites in rats, whereas oxidation of the methyl group to benzoic acid occurred commonly in mice. The identities of 2,4-DAT-related chemicals in the urine were 2,4-diacetylaminobenzoic acid, 4-acetylamino-2-aminobenzoic acid, 2,4-diaminobenzoic acid, 2-acetylamino-4-aminotoluene, 4-acetylamino-2-aminotoluene, 2,4-diacetylaminotoluene, α-hydroxy-2,4-diacetylaminotoluene, α-hydroxy-2,4-diaminotoluene, and an unidentified nitroso and/or benzylic derivative, as well as the parent 2,4-DAT, in both mice and rats. In general, mice have lower levels of the aminotoluene metabolites (2-acetylamino-4-aminotoluene, 4-acetylamino-2-aminotoluene, and 2,4-diacetylaminotoluene) than rats. This could be explained by a greater N-acetyltransferase activity in the liver and a greater capacity for methyl oxidation, which favors the formation of benzoic acid metabolites (2,4-diacetylaminobenzoic acid and 2,4-diaminobenzoic acid) in mice. The authors also hypothesized that the greater ability of 4-acetylamino-2-aminotoluene and 2,4-diacetylaminotoluene to form N-hydroxylamines or N-hydroxylamides (considered the reactive metabolites for carcinogenic aromatic amines and amides) in rats than mice (Miller and Miller, 1969) might be the reason for the greater carcinogenic activity of 2,4-DAT in rats as compared to that in mice.

After administration of 2,4-DAT (99 percent purity) via oral gavage at a dose of 50 mg/kg to female Wistar rats, female New Zealand White rabbits, and female Dunkin-

Harvey guinea-pigs, small amounts of unchanged 2,4-DAT (0.1 to 1.3 percent of the administered dose) and a 4-acetyl derivative (0.7 to 2.3 percent of the administered dose) were detected in the urine (Waring and Pheasant, 1976). Rats also excreted trace levels of 2-acetyl (0.1 percent of the administered dose) and diacetyl derivatives (0.1 percent of the administered dose) in the urine. Other phenolic metabolites identified in the urine of these species included 5-hydroxy-2,4-DAT (the major phenolic metabolite observed in all three species), 3-hydroxy-2,4-DAT, and 6-hydroxy-2,4-DAT (only found in rats). Additionally, two acetyl derivatives of the phenolic metabolites were identified as 3-hydroxy-4-acetylamino-2-aminotoluene and 5-hydroxy-4-acetylamino-2-aminotoluene (Waring and Pheasant, 1976). Treatment of rabbits with phenobarbitone (to induce hydroxylating enzymes) increased the amount of free phenols and glucuronides excreted but decreased the excretion of acetylated compounds. None of these species produced aminobenzoic acid metabolites in the urine. The authors concluded that *p*-hydroxylation was the predominant metabolic pathway for 2,4-DAT in rats, rabbits, and guinea-pigs. Rabbits and rats were also shown to hydroxylate the *m*-position (3-position) ring carbon of 2,4-DAT, despite the steric hindrance of the two aromatic amino groups located adjacent to the hydroxylation position. These phenolic metabolites could either cause tissue damage or undergo glucuronidation for fecal elimination. Acetylation of the amino group occurred preferentially on the 4-amino group in rats, rabbits, and guinea-pigs.

2,4-DAT (in vitro)

2,4-DAT was incubated with ^{14}C-acetyl-CoA and liver cytosol preparations obtained from various species: Syrian golden male hamsters, male guinea pigs, male albino rabbits, male and female Swiss mice, male and female F344 rats and one mongrel dog (Glinsukon *et al.*, 1975). 4-Acetylamino-2-aminotoluene was identified as a metabolite produced in incubations with liver cytosol from all species examined except dog. Trace levels of 2,4-diacetylaminotoluene were also formed in incubations with liver cytosol from hamsters, guinea-pigs, rabbits and mice, but not in rats or dog. In a separate follow-up study, 2-acetylamino-4-aminotoluene was observed in incubation with hamster cytosol at trace amounts (Glinsukon *et al.*, 1976). In the multi-species experiment, N-acetylation of 2,4-DAT varied across tissues and species. In hamsters, the highest cytosolic N-acetyltransferase activity was present in the liver, followed by the kidneys, intestinal mucosa, and lungs. The order of cytosolic N-acetyltransferase activity in rabbit tissues (from highest to lowest) was liver, intestinal mucosa, lungs, and kidneys. Tissues with high enzyme levels produced both 4-acetylamino-2-aminotoluene and 2,4-diacetylaminotoluene. Hepatic N-acetylation activities differed across species, ranging from the highest to lowest in the following order: hamster, guinea pig, rabbit, mouse, rat, and finally no activity in dog. Therefore, incubation of 2,4-DAT with dog

liver cytosol did not generate any N-acetylated metabolites of 2,4-DAT. There were some sex differences in the levels of N-acetyltransferase activity, with female mice having slightly higher activity than male mice, and male rats having slightly higher activity than female rats.

In a separate *in vitro* study, Sayama *et al.* (2002) examined the formation of N-acetylated metabolites of 2,4-DAT in the presence of various 2,4-DAT metabolites, in liver cytosol prepared from male Wistar rats. Transfer of the acetyl moieties between 2,4-DAT and N-acetylated metabolites was observed. The authors concluded that the formation of 2,4-diacetylaminotoluene during the metabolism of 2,4-DAT depended on the N-acetylation of 4-acetylamino-2-aminotoluene, and the formation of 2-acetylamino-4-aminotoluene depended either on the N-deacetylation of 2,4-diacetylaminotoluene or on the acetyl transfer of 2,4-diacetylaminotoluene. These acetylation and deacetylation reactions are shown in Figure 2 below.

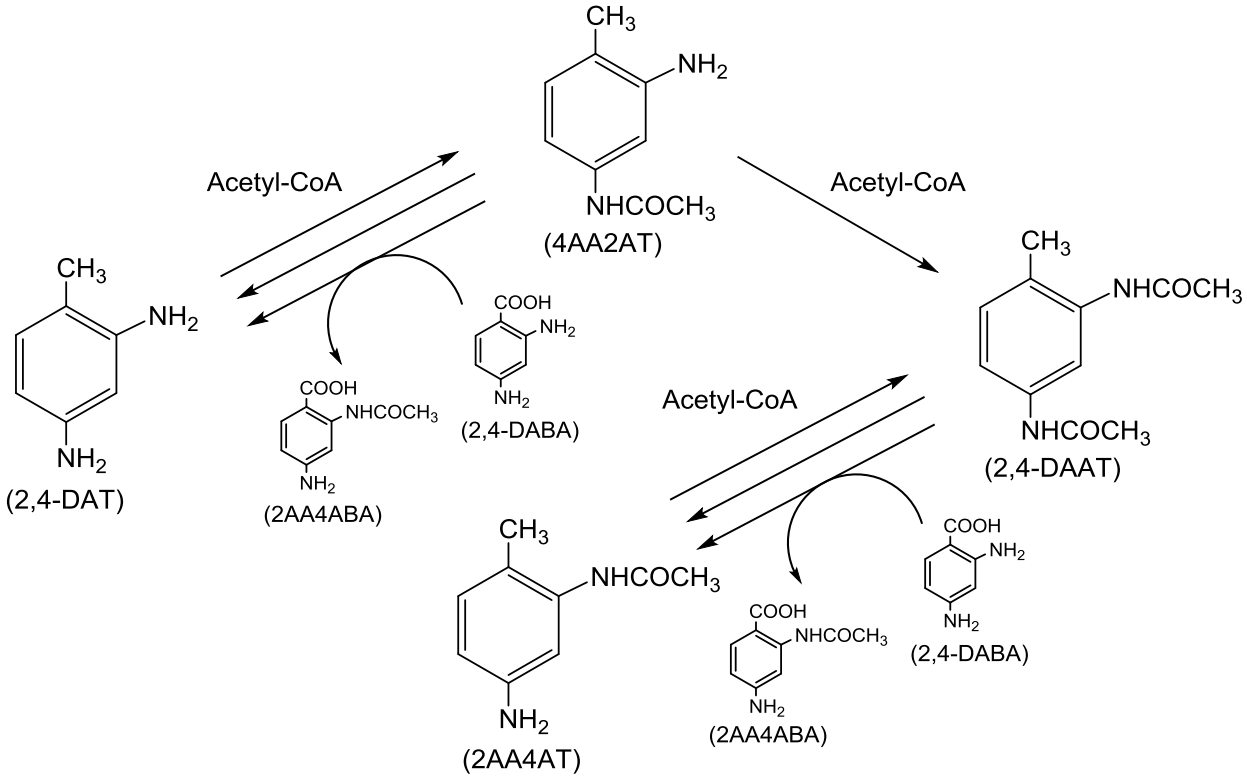


Figure 2. N-acetylation, N-deacetylation, and N-acetyl transfer reactions of 2,4-DAT in Male Wistar rat liver cytosol fraction, as presented by Sayama *et al.* (2002)

2,5-DAT (in vivo)

Six female Kyoto rats (animal model for slow acetylation) were given a single oral gavage dose of 2.5 or 25 mg/kg ^{14}C-radiolabeled 2,5-DAT sulfate [radiolabeled ring-U-^{14}C] (Wenker, 2005b, as reported in SCCS, 2012) . In a separate experiment, another group of four female Kyoto rats received an *i.v.* injection of 3.5 mg/kg 2,5-DAT sulfate (Wenker, 2005a, as reported in SCCS, 2012, pp. 51-52) . Three metabolites identified in the urine following oral administration were 2,5-diacetylaminotoluene [N,N'-diacetyl-toluene-2,5-diamine] and two unidentified mono-N-acetylated metabolites. The major metabolite detected in the urine of *i.v.* injected animals was 2,5-diacetylaminotoluene; a second, unidentified metabolite was also detected. 2,5-Diacetylaminotoluene was also found in the feces of the *i.v.* injected rats (Wenker 2005a, as reported in SCCS, 2012).

2,5-DAT (in vitro)

Phase I and Phase II metabolism of 2,5-DAT sulfate was examined *in vitro* using hepatocytes harvested from male SD rats and male ICR/CD-1 mice. Rats were designated as rapid metabolizers, while the CD mice were considered a mixed population of rapid and slow metabolizers. 2,5-DAT was extensively metabolized by hepatocytes of both species, with rat metabolism surpassing mouse and human metabolism (discussed in Section 3.3.1.1). Only the mono-N-acetylated metabolite of 2,5-DAT was detected in these studies, and both NAT1 and NAT2 enzymes were found to be involved in the acetylation. Mouse hepatocytes also showed extensive hydroxylation of 2,5-DAT (Krebsfanger, 2003, as reported in SCCS, 2012).

2,6-DAT (in vivo)

Male F344 rats received a single oral gavage dose of 10 mg ^{14}C-radiolabeled 2,6-DAT and metabolites excreted in the urine within the first 24 hours were examined (Cunningham *et al.*, 1989). The urinary metabolites observed were 3-hydroxy-2,6-diaminotoluene, 5-hydroxy-2-acetylamino-6-aminotoluene, 2-acetylamino-6-aminotoluene, and 2,6-diacetylaminotoluene. No parent compound was present in the urine.

Elimination

Urinary excretion is the main route of elimination of DATs regardless of the route of administration. Several *in vivo* animal studies examining the elimination of DATs following various routes of administration were available in the literature:

2,4-DAT

In male B6C3F mice, following *i.p.* injection of ^{14}C-radiolabeled 2,4-DAT, about 50 percent of body burden radioactivity was present in the urine of animals sacrificed at one to 24 hours (Unger *et al.*, 1980). 2,4-DAT and/or its metabolites were rapidly eliminated in the urine within hours of *i.p.* injection in mice. No radioactivity was detected in the feces for the first two hours. Radioactivity in the feces accounted for two percent of the body burden at three hours and sharply rose to 20 and 22 percent at 16 and 24 hours following injection, respectively, demonstrating that 2,4-DAT and/or its metabolites were redistributed into the GI tract and then eliminated via fecal deposition. A small percentage of the administered dose, 1.25 percent, was eliminated in expired air over the 24 hour period following *i.p.* injection.

Grantham *et al.* (1979) conducted a 2,4-DAT metabolism study on male Fischer rats and male Swiss mice using *i.p.* injection. Elimination of radioactivity from all tissues (with the exception of stomach and intestines) followed a biphasic pattern. Half-lives for liver, kidney, and blood were 0.89, 0.43, and 1.51 hours respectively, for the fast elimination phase. The slow elimination phase had half-lives of 11.7, 9.1, and 12.6 hours for liver, kidney, and blood. Urinary excretion was the major elimination pathway, accounting for 75 percent of the administered dose in mice in the first eight hours and 50 percent of the administered dose in rats. Excretion via the feces was greater in rats (slightly above 15 percent of the injected dose on day two) than in mice (less than two percent of the injected dose on day two).

In another ^{14}C-radiolabeled 2,4-DAT *i.p.* injection study, Reddy *et al.* (1986) reported that about 70 percent of the radioactivity was excreted in the urine collected from adult male F344 rats 24 hours after dosing. This study also looked at the effect of pretreatment with microsomal enzyme inducers on urinary excretion rates. The lowest amount of radioactivity excreted was 62 percent from animals pretreated with 3-methyl-cholanthrene and the highest amount was 74.1 percent from the saline control animals.

In the oral gavage and *i.v.* infusion studies in male F344 rats conducted by Timchalk *et al.* (1994), urinary excretion half-lives of 2,4-DAT were eight hours for the 60 mg/kg oral dose and five hours for the 3 mg/kg oral or *i.v.* doses. Over a 48-hour period, similar urinary excretion was observed among all the treatment groups: slightly above 60 percent of the applied dose in the two orally dosed groups and 73 percent in the *i.v.* infusion group. The percentage of radioactivity recovered in the feces was similar among all the treatment groups, with recovery of 31 and 23 percent of the applied doses for the low and high oral dose groups and 20 percent for the *i.v.* infusion group. The

data demonstrate urinary excretion is the main excretion pathway for 2,4-DAT following either oral gavage or *i.v.* infusion.

2,5-DAT

Results from a mass balance study by Wenker (2005b) indicated that a single oral dose of either 2.5 or 25 mg radiolabeled 2,5-DAT sulfate given to Kyoto rats resulted in a cumulative radioactivity recovery of 62.2 and 72.9 percent in the urine by 96 hours for the low- and high-dose groups, respectively. Amounts of radioactivity recovered from feces were 31.4 and 22 percent for the low- and high-dose groups, respectively (Wenker, 2005b, as reported in SCCS, 2012). In a separate experiment, groups of female Kyoto rats received a single *i.v.* dose of 2.5 mg 2,5-DAT sulfate/kg bw. Urinary excretion in rats accounted for 54 percent and fecal excretion accounted for 27 percent of the administered dose, with a total of 81 to 87 percent excreted during the 96 hour study period (Wenker, 2005a, as reported in SCCS, 2012).

In the studies conducted by Hruby (1977), rats excreted less than one percent of the *s.c.* applied dose of radiolabeled 2,5-DAT in 24 hours (equivalent to 68 and 38 percent of the absorbed dose for formulations 1 and 2, respectively), compared to over 70 percent of the administered dose excreted in the urine of rats receiving 2,5-DAT via oral gavage (Hruby, 1977). Urinary excretion was completed within five days. Fecal excretion for both the *s.c.* and oral groups amounted to approximately five percent each day for the first two days and was completed by five days. Approximately seven percent of the applied radioactivity was recovered from the carcass and two percent remained at the application site, five days after *s.c.* injection. Five days after oral administration, approximately one percent of the administered dose was recovered from the GI tract and one percent from the carcass. Taken together, irrespective of the route of administration, 2,5-DAT and/or its metabolites were mainly excreted in the urine in rats. Judging from the low levels of radioactivity recovered in the urine and feces from days two to four, there was little evidence for enterohepatic circulation of 2,5-DAT or its metabolites in rats.

Elimination of 2,5-DAT and/or its metabolites in the study in dogs was highly dependent on the routes of administration (Hruby, 1977). Most of the radioactivity was eliminated in the feces following dermal application of radiolabeled 2,5-DAT and fecal elimination increased from days one to four. In contrast, when 2,5-DAT was administered by *i.v.* infusion for three hours, most of the radioactivity was recovered in the urine and some radioactivity showed up in the feces within the first day, while residual levels were recovered in both the urine and feces from days two to four. This study indicates that fecal elimination of 2,5-DAT or its metabolites is a significant route of excretion in the

dog, especially following dermal application. Moreover, the presence of radioactivity in the feces after *i.v.* infusion and the increased fecal elimination in later days following dermal application suggest that after entering the circulatory system 2,5-DAT and/or its metabolites are distributed to the liver, enter the bile, and are excreted into the GI tract for fecal elimination.

Dermal application of 1.4 g 2,5-DAT sulfate in a non-oxidative gel mix to male dogs for three hours resulted in excretion of less than 0.04 percent of the applied dose in urine over a two-day period (Kiese *et al.*, 1968). In contrast, urinary excretion within the first 24 hours after *i.v* infusion was 60 percent of the total applied dose, while fecal excretion accounted for 19 percent of the total radioactivity.

2,6-DAT

A single dose of 10 mg of 2,6-DAT was given to rats via oral gavage. Urinary excretion was the main pathway with more than 80 percent of the applied dose excreted within 24 hours. Fecal excretion was minor, with approximately 10 percent excreted over three days. No radioactivity was recovered in the exhaled air (Cunningham *et al.*, 1989).

3.3.1.3 Summary

Data on the disposition of DATs mainly come from animal studies, since studies in humans are limited. Among these available studies, three isomers: 2,4-, 2,5-, and 2,6-DAT, and their salts were used in the animal studies via multiple routes of administration, i.e., oral gavage, dermal, *i.v.* infusion, *i.p.* injection, and *s.c.* injection. The human studies consist of primarily dermal application studies of 2,5-DAT (or its salts) in hair dye formulations, with one *s.c.* injection study of 2,5-DAT and one dermal application study of 2,4-DAT. Additional *in vitro* studies using skin models (from human or pig) and subcellular preparations (from liver of humans and animals) are available on 2,4-DAT and/or 2,5-DAT, and provide information on absorption and metabolism. Among the three isomers examined, no noticeable differences in pharmacokinetics can be detected, potentially due to the lack of studies specifically designed to compare across isomers. Therefore, the general discussion on DATs below is based on data from 2,4- (limited), 2,5- (the most studied isomer), and 2,6-DAT.

DATs are rapidly and extensively absorbed following oral ingestion. Levels of the DATs or their metabolites in the blood peaked within an hour, and an absorption efficiency of approximately 70 percent has been noted in rats, the only species examined using radiolabel tracers with oral administration. Dermal absorption of DATs is relatively fast, but generally slower than absorption by the oral route. Blood levels of radioactivity

(from radiolabel on DATs) peaked around two to three hours in humans and between one-half to two hours in animals following dermal application. Dermal absorption efficiency is low and is highly depended on the duration of exposure. Dermal application for 24 hours resulted in 24 and 54 percent absorption in humans and monkeys, respectively, in one study on 2,4-DAT. However, absorption ranged from < 1 to < 10 percent in non-primate animal studies with exposure durations of 30 minutes to three hours. The six DAT isomers have log Kow (log value of the ratio of concentration in octanol phase to concentration in aqueous phase) ranging from 0.14 to 0.71 (Table 1). This suggests that dermal uptake of the individual isomers can be different. 2,4-, 2,5-, and 2,6-DAT have similar and low log Kow values (0.14 to 0.16), indicative of a lower lipophilicity than the other three isomers, and thus are expected to have lower rates of dermal uptake.

Irrespective of the route of administration, DATs are well distributed throughout the body with no noticeable accumulation in organs. DATs and/or their metabolites have been detected in the blood, liver, kidneys, lymph nodes, eyes, skin, muscle, adrenal gland, small and large intestine, spleen, and testes. The carcass and GI tract tissues contained very low levels upon completion of the animal studies, usually at four to five days after DAT administration.

N-acetylation, methyl-oxidation, or ring hydroxylation of DATs has been demonstrated in human and animal studies. DATs are metabolized to mono-N-acetyl and N,N'-diacetylamino metabolites by N-acetyltransferases*, in vivo* in humans and *in vitro* using human hepatic cytosolic preparations. In animals, predominantly the mono-N-acetylated metabolites, with trace levels of N,N'-diacetylated metabolites of DATs have been reported. N-acetylation occurs preferentially on the non-*o*-amino group of DATs, i.e., N-acetylation on the amino group distant to the methyl group. Diacetylated metabolites have been found in animals with a high capacity for N-acetylation, e.g., hamsters. Trace levels of a 2-acetylated metabolite have been detected in a 2,4-DAT study and this was proposed to be a deacetylation product of the 2,4-diacetylaminotoluene. The hypothesis is supported by an *in vitro* metabolite study using a rat hepatic cytosolic preparation. No acetylated metabolites were detected *in vivo* in dogs, suggesting the absence of acetylation or low acetylation capacity in dogs.

Methyl-oxidation of DATs results in the formation of N-acetylated aminobenzoic acids in mice. Mice have a greater capacity for methyl oxidation and a greater N-acetyltransferase activity in the liver, which favors the formation of benzoic acid metabolites. Additionally, hydroxylation (oxidation) of the methyl group generates α-hydroxy metabolites. α-Hydroxy-2,4-diacetylaminotoluene and

α-hydroxy-2,4-diaminotoluene have been reported in both mice and rats exposed to 2,4-DAT.

Phenolic metabolites are generated predominantly from ring hydroxylation potentially mediated by microsomal enzymes. This class of metabolites has not been reported in the limited in vivo or in vitro human studies. In rats exposed to 2,4-DAT, hydroxyl-metabolites like 5-hydroxy-2,4-DAT (major metabolite) and 5-hydroxy-4-acetylamino 2 aminotoluene were detected in the urine, indicative of a preferential hydroxylation at the p-position to the 2-amino group. Evidence of the hydroxylation at *m*- and *o*-positions to the amino groups of 2,4-DAT can also occurr in rats receiving dermal application of 2,4-DAT, with metabolites 6-hydroxy-2,4-DAT (hydroxylation at *m*-position to both the 2- and 6-amino groups), 3-hydroxy-2,4-DAT and 3-hydroxy-4-acetylamino-2-aminotoluene (*o*-position to both the 2- and 6-amino groups, minor metabolites).

Overall, based on limited studies, the metabolites identified in humans are 4-acetylamino-2-aminotoluene (from 2,4-DAT) and 2,5-diacetylaminotoluene (from 2,5-DAT).

Metabolites definitively[13] identified in animals are:

- 2,4-DAT (extensively studied): 2,4-diaminobenzoic acid, 4-acetylamino-2-aminobenzoic acid, 2,4-diacetylaminobenzoic acid, 2-acetylamino-4-aminotoluene, 4-acetylamino-2-aminotoluene, 2,4-diacetylaminotoluene, α-hydroxy-2,4-diacetylaminotoluene, α-hydroxy-2,4-diaminotoluene, 3-hydroxy-2,4-diaminotoluene, 5-hydroxy-2,4-diaminotoluene, 6-hydroxy-2,4- diaminotoluene, 3-hydroxy-4-acetylamino-2-aminotoluene, 5-hydroxy-4-acetylamino-2-aminotoluene
- 2,5-DAT (two *in vivo* and one *in vitro* study): 2,5-diacetylaminotoluene (*in vivo*)
- 2,6-DAT (one *in vivo* study): 3-hydroxy-2,6-diaminotoluene, 5-hydroxy-2-acetylamino-6-aminotoluene, 2-acetylamino-6-aminotoluene, and 2,6-diacetylaminotoluene

Among all these identified metabolites, three have been shown to be mutagenic in bacterial systems: 4-acetylamino-2-aminotoluene (from 2,4-DAT), and 5-hydroxy-2-acetylamino-6-aminotoluene and 2,6-diacetylaminotoluene (both from 2,6-DAT).

[13] Metabolites observed, but not definitively characterized (i.e., metabolites of unknown structure), are not included

Urinary excretion of DATs (and/or their metabolites) is an important route of elimination in both humans and animals. This clearance of DATs (and/or metabolites) via the urine follows first order kinetics and is generally completed within a 24-hour period after DAT administration. Excreted amounts ranged from 54 to 92 percent of the DAT body burden in a 24- or 48-hour period, depending upon the experimental design and the route of administration. Fecal deposition plays a minor role in excretion of DATs and their metabolites in most of the species examined, but can be significant in slow or non-acetylating species like dogs. The parent DATs, N-acetylated metabolites, and phenolic metabolites have been identifed in the urine. Phenolic metabolites may undergo glucuronidation and excretion via the feces, but studies characterizing this conjugation pathway are not available in the literature.

3.3.2 Genotoxicity

The genotoxicity of five of the DAT isomers (all but 3,5-DAT) has been investigated in one or more test systems. The available genotoxicity findings for the DAT isomers are summarized briefly in Table 7.

Table 7. Summary of genotoxicity findings for the DAT isomers

Isomer	Bacteria or Yeast Assays	*In Vitro* Mammalian Cell Assays	*In Vivo* Assays
2,3-DAT	+ Reverse mutation	NT	-
2,4-DAT	+ Reverse mutation, DNA damage, chromosomal recombination	+ Mutation, DNA damage, Covalent binding to DNA, DNA adduct, unscheduled DNA synthesis (UDS), micronucleus, intrachromosomal recombination, sister chromatid exchange (SCEs), and chromosome aberration (CA)	+ Mutation, DNA damage, DNA adduct, UDS, and micronucleus
2,5-DAT	+ Reverse mutation, DNA damage	+ UDS, DNA damage, CA	+ DNA damage in rat stomach, DNA synthesis inhibition in mouse testes
2,6-DAT	+ Reverse mutation, DNA damage, chromosomal recombination	+ Mutation, Covalent binding to DNA, UDS, MN, SCEs, CA	+ DNA damage in rat liver, DNA adduct in rat liver, MN in rat bone marrow, UDS in rat liver, DNA synthesis inhibition in mouse testes
3,4-DAT	+/-[1] Reverse mutation	NT	+ DNA synthesis Inhibition in mouse testes
3,5-DAT	NT	NT	NT

NT, not tested
[1] Weakly positive in one study in *Salmonella*

The available genotoxicity findings for each of the DAT isomers, including the 2,4-isomer, which is already listed individually as causing cancer under Proposition 65, are discussed in more detail below.

2,3-DAT

The genotoxicity of 2,3-DAT was investigated in one study in *Salmonella typhimurium* and in one *in vivo* study in mice.

2,3-DAT was tested in the *Salmonella typhimurium* reverse mutation assay in strain TA98, which detects frameshift mutations, in the presence of Aroclor 1254-induced, DAT-induced (authors did not specify which DAT isomer), and non-induced rat liver microsomes (Cheung *et al.*, 1996). As shown in Table 8, 2,3-DAT was weakly positive under all treatment conditions. No significant differences in mutagenicity were observed with the use of liver microsomes from non-induced rats as compared to rats induced with Aroclor 1254 or DAT.

Table 8. Mutagenicity studies of 2,3-DAT in *Salmonella* (Cheung *et al.*, 1996)

Strain	Conc. Tested	Metabolic Activation System[1]	Results	
			- Metabolic activation	+ Metabolic activation
TA98	2-20 µg/plate	Rat hepatic microsomes, non-induced	NT	+[1]
		Rat hepatic microsomes, Aroclor 1254 induced	NT	+[1]
		Rat hepatic microsomes, DAT induced	NT	+[1]

NT: Not Tested
[1] Weakly positive

2,3-DAT was tested *in vivo* in a mouse chromosomal aberration (CA) assay by Mikstacki (1985). Treatment with 2,3-DAT did not induce CA in the bone marrow or in transplanted Ehrlich ascites tumor cells in CFW inbred mice (Table 9).

Table 9. *In vivo* genotoxicity studies of 2,3-DAT (Mikstacki, 1985)

Species/ Strain/ Sex	Tissues Analyzed	Dose	Route	Results
CFW inbred mice, male	Bone marrow	57.1 mg/kg, 142.8 mg/kg	*i.p.*[1]	-
	Transplanted Ehrlich ascites tumor cells			-

[1] Animals were injected *i.p.* with 0.4 ml ascites fluid (containing $2X10^7$ cells) 24 hours before treatment with 2,3-DAT.

2,4-DAT

The genotoxicity of 2,4-DAT has been studied in a number of bacterial assays, one study in yeast, multiple *in vitro* mammalian cell assays and cell free systems, an *in vivo* assay in *Drosophila*, and several *in vivo* assays in rats and mice. The findings from these genotoxicity studies are summarized briefly in Table 10.

Table 10. Summary of genotoxicity findings on 2,4-DAT

Assay	Organism/Cell Type	Results	Metabolic Activation needed?
Bacterial Assays			
Gene Mutation	*Salmonella typhimurium*	+	yes
DNA Damage (umu test)	*Salmonella typhimurium*	+	yes
Yeast Assays			
Intrachromosomal Recombination (in yeast)	*Saccharomyces cerevisiae* RS112	+	no
***Drosophila* and Mammalian Assays**			
in vitro			
Mutation	Mouse lymphoma cells	+ (*tk* locus) - (*hgprt* locus)	no
	Chinese hamster ovary (CHO) cells	+ (*tk* locus) - (*hgprt* locus)	no
DNA Damage	Human hepatocellular carcinoma HepG2 cells	+	endogenous
	3-D Reconstructed Human epidermal cells	+	endogenous
DNA Adduct Formation (cell-free)	Calf thymus DNA	+	only tested with activation
Covalent Binding to DNA	Primary rat hepatocytes	+	endogenous
Micronucleus (MN)	HepG2 cells	+	endogenous
Chromosome Aberration (CA)	CHO cells	+	no
Sister Chromatid Exchange (SCE)	CHO cells	+	no
Unscheduled DNA Synthesis (UDS)	Primary rat hepatocytes	+	endogenous
	Primary human hepatocytes	+	endogenous
	HepG2 cells	+	endogenous
In vivo			
Mutation	*Drosophila melanogaster*	+	endogenous

Assay	Organism/Cell Type	Results	Metabolic Activation needed?
	Big Blue® B6C3F1 transgenic mice	+ (liver)	
	Muta® transgenic mice	+ (liver and kidney), - (skin at dose site)	
	F344 *gpt* delta transgenic rats	+	
DNA Damage	CD-1 mice	+ (liver, kidney, and lung) - (spleen and bone marrow)	
	SD rats	- (liver)	
DNA Adduct Formation	F344 rats	+ (liver, mammary gland, kidney, lung) - (spleen)	
DNA Fragmentation	SD rats	+ (liver)	
MN	F344 rats	+ (liver) - (bone marrow erythrocytes)	
	F344 *gpt* delta transgenic rats	- (peripheral blood cells)	
	F344/DuCrl Crlj rats	+ (liver)	
	SD rats	+ (liver)	
	Crl:CD (SD) rats	+ (liver)	
Dominant Lethal Assay	DBA/2J mice	-	
SCE	Swiss mice	+ (bone marrow)	
UDS	Fischer rats (hepatocytes)	+	
	SD rats (thoracic aortic smooth muscle cells)	+	

These genotoxicity findings for 2,4-DAT are presented in more detail below in Table 11 (studies in bacteria and yeast), Table 12 (*in vitro* studies in mammalian cells and cell free systems), and Table 13 (*in vivo* studies in *Drosophila* and rats and mice).

As shown in Table 11, 2,4-DAT has been tested in the *Salmonella* reverse mutation assay in numerous studies, using several different test strains, including TA97, TA98, TA100, TA1538, YG1006, YG1024, and YG1029. Strains YG1006, YG1024 and YG1029 express higher levels of N-acetyltransferase activity and are derived from

TA1538, TA98 and TA100, respectively. Strains TA97, TA98, TA1538, YG1006, and YG1024 detect frame shift mutations, and strains TA100, TA1535, NM2000, NM2009, and YG1029 detect G/C base pair substitution mutations.

Positive results for reverse mutation have been reported in at least one study for all but one of the *Salmonella* strains tested in the presence of metabolic activation, and negative results have been reported in the absence of metabolic activation. 2,4-DAT was not mutagenic in the one study conducted in strain TA97, which tested prostaglandin synthase H/arachidonic acid as the metabolic activation system (Pan *et al.*, 1992). These results indicate that 2,4-DAT requires metabolic activation and causes both base pair and frame shift mutations in *Salmonella*, although it appears to be more potent in inducing frameshift (e.g., in TA98 and TA1538) than base pair substitution mutations (e.g., TA100) (Pienta *et al.*, 1977; Greene *et al.*, 1979; Shahin *et al.*, 1980; Parodi *et al.*, 1981).

With S-9 metabolic activation, 2,4-DAT induced DNA damage in *Salmonella* in the umu test in strains TA1535 and NM2000, but not in strain NM2009 (Oda *et al.,* 1995; Yasunaga *et al*., 2006). The umu test detects DNA damage by assessing induction of the umu gene that is part of the bacterial response to DNA damage (Table 11).

2,4-DAT was positive in the *Saccharomyces cerevisiae* intrachromosomal (DEL) recombination assay in the presence or absence of rat liver S-9 (Brennan and Schiestl, 1997) (Table 11). The DEL assay detects homologous intrachromosomal recombination that leads to genomic deletions.

Table 11. Genotoxicity studies of 2,4-DAT in *Salmonella* and *Saccharomyces*

Strain	Concentration Tested	Activation System	Results		Reference
			-S-9	+S-9	
Frame Shift Sensitive Assays in *Salmonella*					
TA97	Up to 100 mM[1]	Prostaglandin Synthase H (PSH)/Arachidonic acid activation system	NT	-	Pan *et al.* (1992)
TA98	Up to 100 mM[1]	PSH/Arachidonic acid activation system	NT	-	Pan *et al.* (1992)
TA98	10-500 µg/plate	Rat liver S-9, Aroclor 1254 induced	-	+	Pienta *et al.* (1977)
TA98	Unspecified	Rat liver S-9, Aroclor 1254 induced or non-induced; or mouse liver S-9, non-induced	NT	+[2]	Greene *et al.* (1979)
TA98	5-1000 µg/plate	Rat liver S-9, Aroclor 1254 induced	-	+	Shahin *et al.* (1980)
TA98	1 µg crude crystal/plate	Rat liver S-9, polychlorobiphenyl (PCB) induced; 2,4-DAT was oxidized by H_2O_2	-	+	Watanabe *et al.* (1987)
TA98	10 µg/plate	Rat liver S-9, PCB induced	-	+	Watanabe *et al.* (1989)
TA98	2 mM	Rat liver S-9, PCB induced	-	+	Kami *et al.* (1997)
TA98	0.5-5 µg/plate	Rat liver microsomes, Aroclor 1254 induced	NT	+[3]	Cheung *et al.* (1996)
		Rat liver microsomes, DAT (not specified) induced	NT	+[4]	
		Rat liver microsomes, non-induced	NT	+[4]	
TA98	50-200 µg/plate	Rat liver microsomes, benzo[a]pyrene induced	NT	+[3]	Cheung *et al.* (1996)
		Rat liver microsomes, induced[5]	NT	+[4]	
TA98	Up to 5 mg/ml	Rat liver S-9, phenobarbital (PB) and β-naphthoflavone (β-NF) induced	-	-	Yasunaga *et al.* (2006)
TA98	Up to 2.5 mM	Rat liver microsomes, Aroclor 1254 induced	NT	+	Pan *et al.* (1992)
TA98	50-200 µg/plate	Rat liver microsomes, non-induced	NT	+[4]	Cheung *et al.* (1996)
TA98	100-3333 µg/plate	Mouse liver S-9, PB/β-NF induced	NT	+	George and Westmoreland (1991)
TA98	50-200 µg/plate	Liver S-9, species unspecified	NT	+	Parodi *et al.* (1981)
TA98	100 µg/plate	Liver S-9, species unspecified	NT	+	Hirayama *et al.* (1985)

Strain	Concentration Tested	Activation System	Results		Reference
			-S-9	+S-9	
TA98	Up to 2500 µg/plate (S-9-) and about 700 µg/plate (S-9+)	Liver S-9, species unspecified	-	+	Toyoda-Hokaiwado *et al.* (2010)
TA98	5-1000 µg/plate	None	-	NT	Mori *et al.* (1982)
TA1538	Up to 100 µg/plate	Rat liver S-9, Aroclor 1254 induced	NT	+	Ames *et al.* (1975)
TA1538	100 µg/plate	Rat liver S-9, Aroclor 1254 induced	NT	+	McCann *et al.* (1975)
TA1538	10-500 µg/plate	Rat liver S-9, Aroclor 1254 induced	-	+	Pienta *et al.* (1977)
TA1538	Up to 1000 µg/plate	Rat liver S-9, non-induced	NT	+[4]	Aune *et al.* (1979)
		Rat liver S-9, PB induced	NT	+[3]	
		Rat liver S-9, β-NF induced	NT	+[3]	
TA1538	5-1000 µg/plate	Rat liver S-9, Aroclor 1254 induced	-	+	Shahin *et al.* (1980)
TA1538	10 µg/plate	Rat or mouse liver microsomes, β-NF induced or non-induced	NT	+	Dybing and Thorgeirsson (1977)
TA1538	300 or 1000 µg/plate	Rat liver S-9, β-NF induced or non-induced	-	+[6]	Furlong *et al.* (1987)
YG1006[7]	Up to 100 mM[1]	PSH/Arachidonic acid activation system	NT	-	Pan *et al.* (1992)
YG1024[7]	Up to 100 mM[1]	PSH/Arachidonic acid activation system	NT	-	Pan *et al.* (1992)
YG1024[7]	Up to 2500 µg/plate (S-9-) and about 200 µg/plate (S-9+)	Liver S-9, species unspecified	-	+	Toyoda-Hokaiwado *et al.* (2010)
G/C Base Pair Substitution Sensitive Assays in *Salmonella*					
TA100	100 µg/plate	Rat liver S-9, Aroclor 1254 induced	NT	+	McCann *et al.* (1975)
TA100	10-500 µg/plate	Rat liver S-9, Aroclor 1254 induced	-	-	Pienta *et al.* (1977)
TA100	5-1000 µg/plate	Rat liver S-9, Aroclor 1254 induced	-	+	Shahin *et al.* (1980)
TA100	Unspecified	Rat liver S-9, Aroclor 1254 induced or non-induced; or mouse liver S-9, non-induced	NT	-	Greene *et al.* (1979)
TA100	Up to 100 mM[1]	PSH/Arachidonic acid activation system	NT	-	Pan *et al.* (1992)
TA100	Up to 5000 µg/plate	Liver S-9, species unspecified	-	+	Toyoda-Hokaiwado *et al.* (2010)
TA100	50-200 µg/plate	Liver S-9, species unspecified	NT	-	Parodi *et al.* (1981)
TA100	100 µg/plate	Liver S-9, species unspecified	NT	+	Hirayama *et al.* (1985)
TA100	5-1000 µg/plate	None	-	NT	Mori *et al.* (1982)
TA100	1-150 µg/ml top agar	None	-	NT	Padda *et al.* (2003)

Strain	Concentration Tested	Activation System	Results		Reference
			-S-9	+S-9	
TA1535/pSK1002, NM2000 (umu Test)[8]	125-1000 µg/ml	Rat liver S-9, PB and β-NF induced	NT	-	Oda *et al.* (1995)
TA1535 (umu Test)	Up to 5 mg/ml	Rat liver S-9, PB and β-NF induced	-	+	Yasunaga *et al.* (2006)
NM2009 (umu Test)[8]	125-1000 µg/ml	Rat liver S-9, PB and β-NF induced	NT	+	Oda *et al.* (1995)
YG1029[7]	Up to 5000 µg/plate	Liver S-9, species unspecified	-	+	Toyoda-Hokaiwado *et al.* (2010)
Mutagenic Urine Test with *Salmonella*					
TA1538	Urine from 2,4-DAT treated rats	Endogenous	+		Cinkotai *et al.* (1978)
Intrachromosomal Recombination Test in *Saccharomyces*					
Saccharomyces cerevisiae RS112	Up to 6 mg/ml	Rat liver S-9, Aroclor 1254 induced	+	+	Brennan and Schiestl (1997)

[1] Concentrations not presented for strains other than TA98

[2] Pre-treatment of mice with 2,4-DAT before preparation of S-9 decreased the mutagenicity

[3] Strongly positive

[4] Weakly positive

[5] Microsomes from rats induced with phenobarbital, isoniazid, dexamethasone, or clofibrate

[6] The number of revertants/plate was significantly higher with β-NF induced S-9 at both doses

[7] The YG strains are derived from standard tester strains: YG1006 from TA1538, YG1024 from TA98, and YG1029 from TA100. All three YG strains have elevated *O*-acetyltransferase activity. The full name of *O*-acetyltransferase is Acetyl-CoA:N-hydroxyarylamine *O*-acetyltransferase, and the corresponding enzyme in higher organisms is *N*-acetyltransferase. YG1006 has elevated activities of both *O*-acetyltransferase and nitroreductase.

[8] These are umu tester strains expressing the fusion gene *umuC'-'lacZ*. Mutagenicity of 2,4-DAT was measured by detecting the levels of β-galactosidase activity induced by the fusion gene. Both NM2000 and NM2009 were derived from TA1535/pSK1002. NM2000 was the *O*-acetyltransferase deficient strain, and NM2009 was the *O*-acetyltransferase overexpressing strain.

2,4-DAT tested positive in multiple *in vitro* mammalian genotoxicity assays and formed DNA adducts in cell free systems (Table 12). Briefly, 2,4-DAT:

- Induced mutations in L5178Y mouse lymphoma cells (in the absence of rat liver S-9) and AT3-2 Chinese Hamster Ovary (CHO) cells (in the presence or absence of S-9) at the autosomal *tk* locus, but not at the sex-linked *hgprt* locus in either cell line
- Damaged DNA (induced DNA-strand breaks) in human HepG2 cells and a three-dimentional (3-D) reconstructed human epidermal cell model
- Formed adducts with calf thymus single-stranded DNA in a cell-free system (in the presence of S-9) and covalently bound to DNA in rat primary hepatocytes
- Induced MN in human HepG2 cells

- Induced CA and sister chromatid exchanges (SCEs) in CHO cells in the presence and absence of rat liver S-9
- Induced unscheduled DNA synthesis (UDS) in human HepG2 cells, primary human hepatocytes, primary rat hepatocytes, and cultured rat thoracic aortic smooth muscle cells

Table 12. *In vitro* genotoxicity studies of 2,4-DAT in mammalian cells and cell-free systems

Test Endpoint/ System	Species /Cell Type	Conc. Tested	Activation system	Results		Reference
				-S-9	+S-9	
Gene Mutation						
Mutation	L5178Y mouse lymphoma cells, *tk* locus	58.5-1000 µg/ml	Rat liver S-9, Aroclor (2:1 mixture of 1242:1254) induced	+	-[1]	Coppinger *et al.* (1984)
	L5178Y mouse lymphoma cells, *hgprt* locus			-	-	
	AT3-2 Chinese Hamster Ovary (CHO) cells, *tk* locus	2-6 mg/ml	Rat liver S-9, Aroclor 1254 induced	+[2]	+[2]	
	AT3-2 CHO cells, *hgprt* locus			-	-	
DNA/ Chromosome Damage						
DNA Damage (Comet Assay)	Human HepG2 cells	1-10 mM	Endogenous	+		Severin *et al.* (2005)
	EpiDerm® Reconstructed 3-D human epidermal cells	Up to 1600 µg/cm^2	Endogenous	+[3]		Reus *et al.* (2013)
DNA Adduct Formation and Covalent Binding to DNA	Calf thymus single-stranded DNA (cell-free)	Up to 200 µg	Rat liver S-9, Aroclor 1254 induced	NT	+[4]	Citro *et al.* (1993)
	Calf thymus DNA (cell-free)	0.5 mM	Rat liver microsomes	-[5]		Aune *et al.* (1979)
	Primary rat hepatocytes	30-300 µM	Endogenous	+[5]		Furlong *et al.* (1987)
			β-NF induced	+[5]		
MN	Human HepG2 cells	1.45-10 mM	Endogenous	+		Severin *et al.* (2005)
CAs	CHO cells	Up to 1370	Rat liver S-9, Aroclor 1254	+	+	Loveday *et al.* (1990)

Test Endpoint/ System	Species /Cell Type	Conc. Tested	Activation system	Results		Reference
				-S-9	+S-9	
		µg/ml	induced			
SCEs	CHO cells	Up to 468 µg/ml	Rat liver S-9, Aroclor 1254 induced	+	+	Loveday *et al.* (1990)
UDS (DNA Repair)						
UDS	Primary rat hepatocytes	0.01-1 mM	Endogenous	+		Bermudez *et al.* (1979)
	Primary rat hepatocytes	0.01, 0.1 or 1 mM	Endogenous	+		Butterworth *et al.* (1989)
	Primary human hepatocytes		Endogenous	+[6]		
	Thoracic aortic smooth muscle cell culture from male SD rats	1-100 µM	Endogenous	+		Ramos *et al.* (1991)
	Human HepG2 cells	0.005-5 mM	Endogenous	+		Severin *et al.* (2005)

[1] Weakly positive at some doses, but no dose response
[2] Positive at higher doses in multiple experiments
[3] Overall evaluation of multiple experiments from multiple laboratories.
[4] DNA adduct
[5] Covalent binding to DNA
[6] Positive in two cases, negative in one case.

Positive *in vivo* evidence of 2,4-DAT genotoxicity includes mutation (in mice, rats, and *Drosophila)*, DNA damage (in rats and mice), and DNA adduct, UDS, DNA fragmentation, and micronuclei (MN) (in rats). 2,4-DAT did not induce dominant lethal mutations in mice.

As shown in Table 13, 2,4-DAT was genotoxic in several *in vivo* assays. Briefly, 2,4-DAT:

- Induced mutations in *Drosophila melanogaster*, causing dominant mutations and sex-linked recessive mutations in F_1 and F_2 offspring, respectively, and in the liver and kidneys in mice and the liver in rats.
- Damaged DNA (induced DNA-strand breaks) in rats and mice in multiple tissues (brain, stomach, colon, kidneys, and liver in rats, and stomach lung, liver, and kidneys in mice).
- Formed DNA adducts in rats in the liver, mammary gland, mammary fat pads, kidneys, and lungs, but not in T-lymphocytes from the spleen, and did not covalently bind to liver DNA

- Induced UDS in rat liver in one of two studies
- Induced MN in rat liver in five studies and in rat bone marrow cells in one of four studies. MN were not induced in rat or mouse peripheral blood cells.
- Induced SCEs in mouse bone marrow cells
- Inhibited DNA synthesis in mouse testes
- Did not cause dominant lethal mutations in mice

Table 13. *In vivo* genotoxicity studies of 2,4-DAT

Endpoint	Species /Strain/Sex	Tissues Analyzed	Dose	Administration Route	Results	Reference
Mutation	*Drosophila melanogaster*	DNA in F_1 (dominant *Minute* and rDNA mutations)	5-20 mM	Injection into the haemocoel of adult males	+	Fahmy and Fahmy (1977)
		DNA in F_2 (sex-linked recessive mutations)			+	
	Big Blue® B6C3F1 transgenic mice[1], male	Liver DNA (*lacI* mutations)	1000 ppm for 30 or 90 days	diet	+[2]	Hayward *et al.* (1995)
	F344 rats, female	Spleen T-lymphocytes (*hprt* locus)	40 or 180 ppm for 6 weeks	diet	-	Delclos *et al.* (1996)
	Muta® CD2-lacZ80/HazfBR transgenic mice, male	Skin DNA at dose site (*LacZ* mutations)	200 mg/kg/day for 28 days	dermal	-	Kirkland and Beevers (2006)
		Liver DNA (*LacZ* mutations)			+[3]	
		Kidney DNA (*LacZ* mutations)			+[3]	
	F344 *gpt* delta transgenic rats, male	Liver DNA (*gpt* mutations)	125, 250, or 500 ppm for 13 weeks[4]	diet	+	Toyoda-Hokaiwado *et al.* (2010)
		Liver DNA (Spi^- selection of deletions)			+	
		Kidney DNA (*gpt* mutations)			-	
	F344/NSlc-Tg *gpt* delta transgenic male rats, male	Liver genomic DNA (*gpt* mutations)	10 or 30 mg/kg/day for 28 days	oral gavage	+	Sui *et al.* (2012)
	DBA/2J mice, male	Fetuses (17 days after mating)	80 mg/kg, in two 40 mg/kg dosing	oral gavage or *i.p.*	-	Soares and Lock (1980)
DNA Damage (Comet Assay and Single Strand DNA Elution)	CD-1 mice, male	Liver, kidney, or lung DNA	240 mg/kg for 3 or 24 h	*i.p.* injection	+	Sasaki *et al.* (1997)
		Spleen or bone marrow DNA			-	
	ddY mice, male	Multiple tissues	60 mg/kg	oral gavage	+[5]	Sasaki *et al.* (1999); Sekihashi *et al.* (2002)
	Wistar rats, male		130 mg/kg		+[6]	
	SD rats, male	Liver DNA	25, 50, or 100 mg/kg/day for 29 days	oral gavage	-	Rothfuss *et al.* (2010)

Endpoint	Species /Strain/Sex	Tissues Analyzed	Dose	Administration Route	Results	Reference
	Sprague-Dawley rats, male	Liver DNA	4.1 mmol/kg, harvested after 4 or 24 h	*i.p.* injection	+	Parodi *et al.* (1981)
DNA Adduct Formation and Covalent Binding to DNA[7]	Wistar rats, male	Liver DNA	100 mg/kg	*i.p.* injection	-	Aune *et al.* (1979)
	Fisher 344 rats, male and female	Liver, mammary gland, kidney, and lung	41 to 2046 µmol/kg, harvested after 18 h	*i.p.* injection	+[8]	La and Froines (1992)
		Liver	410 µmol/kg, harvested at various time points (6 h to 14 days)	*i.p.* injection	+	
	Fisher 344 rats, male	Liver, mammary gland, kidney, and lung	50 mg/kg, harvested after 18 h	*i.p.* injection	+[8]	La and Froines (1994)
		Liver	50 mg/kg, harvested at various time points (6 h to 14 days)	*i.p.* injection	+	
		Liver	Single injection of 5 or 50 mg/kg, or multiple injections (3x5 mg/kg or 10x5 mg/kg)	*i.p.* injection	+	
	Fisher 344 rats, male	Liver	125 or 250 mg/kg, harvested after 18 h	*i.p.* injection	+	Taningher *et al.* (1995)[9]
	Fisher 344 rats, female	Liver	10-180 ppm for up to 6 weeks	diet	+	Delclos *et al.* (1996)
		Mammary fat pads			+	
		T-lymphocytes from spleen			-	
	Fisher 344 rats, male	Liver	0.5-250 mg/kg, harvested after 24 h	*i.p.* injection	+	Wilson *et al.* (1996)
UDS	Fisher 344 rats, male	Liver hepatocytes	150 mg/kg in corn oil, harvested after 2 or 12 h	oral gavage	+	Mirsalis *et al.* (1982)
	Either F344 or PVG rats (not specified)	Liver hepatocytes	150 or 300 mg/kg, harvested after 2 or 16 h	Oral gavage	-[10]	George and Westmoreland (1991)
MN	Fisher 344 rats, male and female	Bone marrow polychromatic erythrocytes	50, 100 or 150 mg/kg (ultrasonicated in water)	oral gavage	-	George and Westmoreland (1991)
	PVG rats, male		150, 225, or 300 mg/kg (Ultraturrax homogenized)		+[11]	

Endpoint	Species /Strain/Sex	Tissues Analyzed	Dose	Administration Route	Results	Reference
	BDF_1 mice, male	Peripheral blood reticulocytes	30-240 mg/kg, harvested after 24, 48, or 72 h	*i.p.* injection	-	Morita *et al.* (1997)
	F344 rats, male	Liver hepatocytes	62.5, 125 or 250 mg/kg (2 dosings), or 250 mg/kg (one dosing), sample harvested 3, 4, or 5 days after dosing	oral	+	Suzuki *et al.* (2009)
	SD rats, male		250 mg/kg, harvested 3, 4, or 5 days after dosing		+	
	SD rats, male	Bone marrow polychromatic erythrocytes	100 mg/kg/day for 29 days	oral gavage	-	Rothfuss *et al.* (2010)
	F344/DuCrlCrlj rats, male	Liver hepatocytes	50, 100, or 200 mg/kg (dosed twice at 24 h intervals)	oral gavage	+	Takasawa *et al.* (2010)
			62.5, 125 or 250 mg/kg (dosed twice at 24 h intervals)		+	
	F344 *gpt* delta transgenic rats, male	Peripheral blood cells	125, 250, or 500 ppm for 13 weeks[4]	diet	-	Toyoda-Hokaiwado *et al.* (2010)
	Crl:CD (SD) rats, male	Liver hepatocytes	25 mg/kg/day for 28 days, or 50 mg/kg/day for 5, 14 or 28 days; harvested 24 h after last dosing	oral gavage	+[12]	Narumi *et al.* (2012)
	Crl:CD (SD) rats, male	Bone marrow erythrocytes	50 mg/kg/day for 5 days or 14 days; or 25, 50, or 100 mg/kg/day for 28 days	oral gavage	-	Takasawa *et al.* (2013)
Inhibition of DNA synthesis[1][3]	C57BL/6 × C3H mice, male	Testes	111-375 mg/kg	*i.p.* injection	+	Greene *et al.* (1981)
SCEs	Swiss mice, male	Bone marrow cells	9 or 18 mg/kg, evaluated after 24 h	*i.p.* injection	+	Parodi *et al.* (1983)

[1] Bearing multiple copies of the bacteriophage λ, each carrying a *lacI* gene
[2] Positive at 90 days but not 30 days
[3] Positive when outliers in the control group were excluded
[4] The high dose was reduced to 400 ppm after week 9

[5] Positive in the stomach, liver, and kidney
[6] Positive in the stomach, colon, kidney, and brain
7 All assays in this category examined DNA adduct formation except for Aune *et al.* (1979), which examined covalent binding to DNA
[8] Adduct level: liver>mammary gland>lung>kidney
[9] This paper also briefly mentioned positive DNA adduct findings with 60 and 120 mg/kg 2,4-DAT in female CD-1 mice.
[10] Weakly positive with ultraturrax homogenization at 150 mg/kg and harvested at 16 h; negative at all doses/harvesting time points with ultrasonication
[11] A small, statistically significant increase was seen at the high dose
[12] Positive except the 5-day treatment
[13] The authors stated that “most carcinogens bind to DNA and thereby have the capability of suppressing template activity”, and therefore “any genetically active material capable of reaching the testes might be expected to inhibit testicular DNA synthesis”.

2,5-DAT

The genotoxicity of 2,5-DAT or 2,5-DAT sulfate or 2,5-DAT dihydrochloride has been studied in a number of bacterial assays, *in vitro* mammalian cell assays, and *in vivo* assays in rats and mice.

As shown in Table 14, 2,5-DAT and 2,5-DAT sulfate have been tested in the *Salmonella* reverse mutation assay in numerous studies, using several different test strains, including TA98, 100, 102, 1535, 1537, and 1538. Positive results have been reported in at least one study for all strains tested in the presence of S-9, and negative results generally have been reported in the absence S-9. 2,5-DAT tested weakly positive in the absence of S-9 in TA98 in one study by Zeller and Pfuhler (2014).

In one study, the mutagenicity of 2,5-DAT in *Salmonella* was tested with or without hydrogen peroxide (H_2O_2), because of the common use of 2,5-DAT in hair dye formulations (Ames *et al.*, 1975). The weak mutagenicity of 2,5-DAT increased significantly (about 40 fold) with H_2O_2 pretreatment. This finding is supported by data from another study on hair dyes, where the oxidation of 2,5-DAT by H_2O_2 led to the formation of mutagenic chemicals (Matsuki *et al.*, 1981).

With S-9 metabolic activation, 2,5-DAT induced DNA damage in *Salmonella* in the umu test (Yasunaga *et al.*, 2006) (Table 14).

2,5-DAT was not mutagenic in *Escherichia coli* in the presence or absence of S-9 (Dunkel *et al*., 1985) (Table 14).

Table 14. Genotoxicity studies of 2,5-DAT and its sulfate salt in bacteria

Strain	Concentration Tested[1]	Activation System	Results		Reference
			-S-9	+S-9	
Reverse mutations in *Salmonella typhimurium*					
TA1538	Up to 100 µg/plate	Rat liver S-9, Aroclor 1254 induced	NT	+[2]	Ames *et al.* (1975)
	Up to 100 µg/plate (2,5-DAT was premixed with H_2O_2, before testing)	Rat liver S-9, Aroclor 1254 induced	NT	+[3]	
TA98	Unspecified	Rat liver S-9, Aroclor 1254 induced or non-induced; or mouse liver S-9, non-induced	NT	+[4]	Greene *et al.* (1979)
TA100			NT	-	
TA98	Up to 3333 µg/plate[5]	Rat/Mouse/Hamster liver S-9, Aroclor 1254 induced or non-induced	-	+	Dunkel *et al.* (1985)
TA100			-	-	
TA1535			-	-	
TA1537			-	+[6]	
TA1538			-	+	
TA98	1-3000 µg/plate	Rat liver S-9, Aroclor 1254 induced	-	+	Chung *et al.* (1995)
TA100			-	+	
TA1538	300 or 1000 µg/plate	Rat liver S-9, β-NF induced or non-induced	-	+[7]	Furlong *et al.* (1987)
TA98	2-20 µg/plate	Rat hepatic microsomes, non-induced	NT	-	Cheung *et al*. (1996)
		Rat hepatic microsomes, Aroclor 1254 induced	NT	+[2]	
		Rat hepatic microsomes, DAT (not specified) induced	NT	-	
TA98	Up to 5000 µg/plate[5]	Liver S-9, species unspecified	-	+[8]	Sokolowski (2003), as cited
TA100			-	+	

Strain	Concentration Tested[1]	Activation System	Results		Reference
			-S-9	+S-9	
TA102			-	-	by Burnett *et al.* (2010)
TA1535			-	+	
TA1537			-	+	
TA98	Up to 5 mg/ml	Rat liver S-9, PB and β-NF induced	-	+	Yasunaga *et al.* (2006)
TA98	10 to 5000 µg/plate	Rat liver S-9, Aroclor 1254 induced	-[8]	+	Zeller and Pfuhler (2014)
TA100			-	+	
TA102			-	+	
TA1535			-	+	
TA1537			-	+	
DNA damage in *Salmonella typhimurium*					
TA1535 (umu Test)	Up to 5 mg/ml	Rat liver S-9, PB and β-NF induced	**-**	**+**	Yasunaga *et al.* (2006)
Mutations in *Escherichia coli*					
WP2 *uvrA*	Up to 3333 µg/plate[5]	Rat/Mouse/Hamster liver S-9, Aroclor 1254 induced or non-induced	-	-	Dunkel *et al.* (1985)

NT, not tested

[1] 2,5-DAT free base, unless otherwise specified

[2] Weakly positive

[3] Strongly positive (about 40-fold increase in mutagenicity compared to no pre-mixing with H_2O_2)

[4] Pre-treatment of mice with 2,5-DAT before preparation of S-9 decreased the mutagenicity

[5] 2,5-DAT sulfate

[6] Positive with induced rat S-9 or hamster S-9

[7] The number of revertants/plate was significantly higher with β-NF induced S-9 at both doses

[8] Weakly positive (3-fold at 1000 µg compared to control)

The genotoxicity of 2,5-DAT or 2,5-DAT sulfate has been tested in several *in vitro* mammalian assays, including the mouse lymphoma mutation assay, the UDS assay in rat and hamster hepatocytes, the Comet assay in hamster and human cells, and CA assays in hamster cells (Table 15). 2,5-DAT (or 2,5-DAT sulfate) was positive in each of these assays, with the exception of the lack of mutagenicity observed in the L5178Y mouse lymphoma *tk* locus mutation assay (Table 15).

Table 15. *In vitro* genotoxicity studies of 2,5-DAT and its sulfate salt in mammalian cells

Endpoint	Species/cell type	Concentration Tested[1]	Activation system	Results	Reference
Mutation	L5178Y mouse lymphoma cells (*tk* locus)	1-15 µg/ml without S-9, 10-100 µg/ml with S-9[2]	Rat liver S-9, Aroclor 1254 induced	-	Wollny (1995), as cited by SCCS (2012)
UDS	Primary rat hepatocytes	0.2-100 µM	Endogenous	+	Kornbrust and Barfknecht (1984)
	Primary hamster hepatocytes	0.2-100 µM		+[3]	
DNA damage (Comet assay)	Chinese hamster V79 lung cells	Up to 50 µg/ml	None[4]	+	Zeller and Pfuhler (2014)
	Human keratinocyte HaCaT cells	Up to 120 µg/ml	None	+[3]	
CAs	CHO cells	14-81 µg/ml	None	+[5]	Chung *et al.* (1995)
	Chinese hamster V79 lung cells	2.5-10 µg/ml without S-9, 100-400 with S-9[2]	Rat liver S-9, PB/β-NF induced	+[6]	Schulz (2002), as cited by SCCS (2012)
	Chinese hamster V79 lung cells	2.5-10 µg/ml	None	+	Zeller and Pfuhler (2014)
		100-400 µg/ml	Rat liver S-9, Aroclor 1254 induced	+	

[1] 2,5-DAT free base, unless otherwise specified
[2] 2,5-DAT sulfate
[3] Weakly positive only at higher concentrations
[4] Chinese hamster V79 lung cells do not express endogenous cytochrome P450.
[5] Fewer metaphases for analyses due to severe cellular toxicity at 81 µg/ml
[6] Positive in the presence and absence of S-9

The genotoxicity of 2,5-DAT or 2,5-DAT sulfate or 2,5-DAT dihydrochloride has been tested *in vivo* in rats and mice in a number of different assays, including Comet assays

in mice and rats, the UDS assay in rats, an inhibition of DNA synthesis assay in mice, dominant lethal assays in rats and mice, recessive spot tests in rats, and MN assays in rats and mice (Table 16). 2,5-DAT inhibited DNA synthesis, an effect suspected to be related to DNA binding, in the testes in mice (Greene *et al*., 1981) and the sulfate salt induced DNA damage in the stomach in Wistar rats, as detected in the Comet assay (Sekihashi *et al*., 2002). No other positive *in vivo* genotoxicity findings were reported for 2,5-DAT or its salts (Table 16).

Table 16. *In vivo* genotoxicity studies of 2,5-DAT and its salts

Endpoint	Species/ Strain/Sex	Tissue Analyzed	Dose[1]	Route	Results	Reference
DNA damage (Comet Assay)	ddY mice, male	Multiple tissues	60 mg/kg[2]	oral gavage	-	Sasaki *et al.* (1999); Sekihashi *et al.* (2002)
	Wistar rats, male	Stomach			+[3]	
		Various other tissues			-	
UDS	SD rats, male	Hepatocytes	20-80 mg/kg[2]	oral gavage	-	Getuli (2002); Cinelli (2004) as cited by SCCS (2012)
Inhibition of DNA synthesis[4]	C57BL/6 × C3H mice, male	Testes	40-55 mg/kg[2]	*i.p.* injection	+	Greene *et al.* (1981)
Dominant Lethal Assay	Charles River CD Rats, male	Fetuses	20 mg/kg[5]	*i.p.* injection	-	Burnett *et al.* (1977)
	Mice, strain unspecified	Fetuses	1.5-150 mg/kg for 5 days[6]	dermal	-	Matheson (1978a) as cited by SCCS (2012)
Spot Test	T stock (male) and C57BL/6J (female) mice	coat color spots of offspring	15-1500 mg/kg on days 9, 10, and 11 post fertilization[6]	dermal	-	Matheson (1978b) as cited by SCCS (2012)
	C57BL/10J (male) and C57BL/16J (female) mice	coat color spots of offspring	30 mg/kg	*i.p.* injection	-	Soares and Lock (1980)
	T stock (male) and C57BL/16J (female) mice				-	
MN	CFY rats (SD descendants), male and female	Bone marrow polychromatic erythrocytes	120 mg/kg[2]	oral gavage	-	Hossack and Richardson (1977)
	NMRI mice, male and female	Bone marrow erythrocytes	15-150 mg/kg[2]	oral	-[7]	Volkner (1995) as cited by SCCS (2012)
	Crl:NMRI BR mice, male and female	Bone marrow erythrocytes	25-90 mg/kg[2]	*i.p.* injection	-	Bornatowicz (2002b), as cited by SCCS (2012)

[1] 2,5-DAT free base, unless otherwise specified
[2] 2,5-DAT sulfate
[3] Positive in the stomach at 8 hours harvesting time
[4] The authors stated that "most carcinogens bind to DNA and thereby have the capability of suppressing template activity", and therefore "any genetically active material capable of reaching the testes might be expected to inhibit testicular DNA synthesis".
[5] Male rats received *i.p.* injections of 2,5-DAT three times weekly for eight weeks and then were mated with untreated female mice of the same strain. Fetuses were examined seventeen days after the beginning of the mating period.
[6] 2,5-DAT dihydrochloride
[7] Sufficient exposure of bone marrow cells to 2,5-DAT sulfate was questionable

2,6-DAT

The genotoxicity of 2,6-DAT or 2,6-DAT dihydrochloride has been studied in a number of bacterial assays, one study in yeast, multiple *in vitro* mammalian cell assays, and several *in vivo* assays in rats and mice.

As shown in Table 17, 2,6-DAT has been tested in the *Salmonella* reverse mutation assay in numerous studies, using several different test strains, including TA98, TA100, TA1538, YG1024, and YG1029. Positive results have been reported in at least one study for all strains tested in the presence of metabolic activation, and negative results in the absence of metabolic activation.

2,6-DAT induced DNA damage in *Salmonella* in the umu test in the presence of rat liver S-9 in one study using a strain overexpressing *O*-acetyltransferase (Oda *et al.*, 1995), but not in other strains or studies (Oda *et al.*, 1995; Yasunaga *et al.*, 2004) (Table 17).

2,6-DAT induced intrachromosomal (DEL) recombination in *Saccharomyces cerevisiae* in the absence, but not the presence of S-9 (Brennan and Schiestl, 1997) (Table 17).

Table 17. Genotoxicity studies of 2,6-DAT and 2,6-DAT dihydrochloride in *Salmonella* and *Saccharomyces*

Strain	Concentration Tested[1]	Activation System	Results		Reference
			-S-9	+S-9	
Reverse mutation in *Salmonella typhimurium*					
TA1538	10 µg/plate	Rat liver microsomes, β-NF induced	NT	+	Dybing and Thorgeirsson (1977)
		Rat liver microsomes, non-induced	NT	-	
		Mouse liver microsomes, β-NF induced	NT	+	
		Mouse liver microsomes, non-induced	NT	+	
TA98	Unspecified	Rat liver S-9, Aroclor 1254 induced or non-induced; or mouse liver S-9, non-induced	NT	+[2]	Greene *et al.* (1979)
TA100			NT	-	
TA98	Up to 30 µmol/plate	Rat liver S-9, Aroclor 1254 induced	-	+	Florin *et al.* (1980)
TA100			-	+	
TA98	100 µg/plate	Mouse liver S-9	NT	+	Hirayama *et al.* (1985)
TA100			NT	+	
TA1538	300 or 1000 µg/plate	Rat liver S-9, β-NF induced or non-induced	-	+[3]	Furlong *et al.* (1987)
TA98	300-700 µg/plate	Rat liver S-9, PB and β-NF induced	NT	-	Sayama *et al.* (1989)
TA100			NT	-	
TA98	100-3333 µg/plate	Mouse liver S-9, PB/β-NF induced	NT	+	George and Westmoreland (1991)
TA98	2-20 µg/plate	Rat hepatic microsomes, non-induced	NT	-	Cheung *et al.* (1996)
		Rat hepatic microsomes, Aroclor 1254 induced	NT	+[4]	
		Rat hepatic microsomes, DAT (not specified) induced	NT	-	

Strain	Concentration Tested[1]	Activation System	Results		Reference
			-S-9	+S-9	
TA100	1-150 µg/ml top agar	None	-	NT	Padda *et al.* (2003)
TA98	Up to 2500 µg/plate (S-9-) and about 1300 µg/plate (S-9+)	Liver S-9, species unspecified	-	+	Toyoda-Hokaiwado *et al.* (2010)
YG1024	Up to 2500 µg/plate (S-9-) and about 400 µg/plate (S-9+)		-	+	
TA100, YG1029	Up to 5000 µg/plate		-	+	
DNA damage in *Salmonella typhimurium* (umu test)					
TA1535/pSK1002, NM2000 (umu Test)[5]	125-1000 µg/ml	Rat liver S-9, PB and β-NF induced	NT	-	Oda *et al.* (1995)
NM2009 (umu Test)[5]			NT	+	
TA1535 (umu Test)	Up to 5000 µg/ml[6]	Rat liver S-9, PB or β-NF induced	-	-	Yasunaga *et al.* (2004)
Intrachromosomal recombination test in *Saccharomyces*					
Saccharomyces cerevisiae RS112	Up to 6 mg/ml	Rat liver S-9, Aroclor 1254 induced	+	-	Brennan and Schiestl (1997)

NT, not tested

[1] 2,6-DAT free base, unless otherwise specified

[2] Liver enzyme induction with 2,6-DAT before S-9 preparation did not affect the mutagenicity

[3] The number of revertants/plate was much higher with S-9 from β-NF induced rats than from non-induced rats at the 1000 µg 2,6-DAT/plate test concentration. No significant difference between induced and non-induced S-9 was detected at the 300 µg 2,6-DAT/plate test concentration.

[4] Strongly positive

[5] These are umu tester strains expressing the fusion gene *umuC'-'lacZ*. Mutagenicity of 2,6-DAT was measured by detecting the levels of β–galactosidase activity induced by the fusion gene. Both NM2000 and NM2009 were derived from TA1535/pSK1002. NM2000 was the *O*-acetyltransferase deficient strain, and NM2009 was the *O*-acetyltransferase overexpressing strain.

[6] 2,6-DAT dihydrochloride

The genotoxicity of 2,6-DAT has been tested in several *in vitro* mammalian assays, including the mouse lymphoma mutation assay, covalent DNA binding in primary rat hepatocytes, the UDS assay in rat and human hepatocytes and rat aortic smooth muscle cells, CA assays in hamster cells, an SCE assay in hamster cells, and an MN

assay in hamster cells (Table 18). The 2,6-isomer tested positive in at least one study for each of these genetic toxicity endpoints. Specifically, 2,6-DAT: was mutagenic in L5178Y mouse lymphoma cells at the *tk* locus in the absence of S-9; covalently bound to rat hepatocyte DNA; induced UDS in primary human hepatocytes and in cultured rat thoracic aortic smooth muscle cells; induced CA in CHO cells in the absence of S-9 in two studies; induced CA in Chinese hamster lung cells in the presence or absence of S-9; and induced SCEs and MN in CHO cells in the presence or absence of S-9 (Table 18).

Table 18. *In vitro* genotoxicity studies of 2,6-DAT and 2,6-DAT dihydrochloride in mammalian cells

Endpoint	Assay System	Conc. Tested	Activation System	Results		Reference
				-S-9	+S-9	
Mutation	L5178Y mouse lymphoma cells, *tk* locus	125-1500 µg[1]	None	+	NT	Myhr and Caspary (1991)
Covalent DNA binding	Primary rat hepatocytes	100 µM	Endogenous	+		Furlong *et al.* (1987)
UDS	Primary rat hepatocytes	0.01, 0.1 or 1 mM	Endogenous	-		Butterworth *et al.* (1989)
	Primary human hepatocytes		Endogenous	+ in two cases, - in one case		
	Thoracic aortic smooth muscle cell culture from male SD rats	1-100 µM	Endogenous	+		Ramos *et al.* (1991)
CAs	CHO cells	Up to 1500 µg/mL[1]	Rat liver S-9, Aroclor 1254 induced	+	-	Gulati *et al.* (1989)
	Chinese Hamster Lung cells	250 µg/mL (S-9-), 1500 µg/mL (S-9+)	Rat liver S-9, PCB (KC-400) induced	+	+	Sofuni *et al.* (1990)
	CHO cells	1000 µg/mL (S-9-), 1600 µg/mL (S-9+)	Rat liver S-9, Aroclor 1254 induced	+	-	
SCEs	CHO cells	Up to 500 µg/mL[1]	Rat liver S-9, Aroclor 1254 induced	+	+[2]	Gulati *et al.* (1989)
MN	CHO cells	10-450 µg/mL (S-9-), 100-1200 µg/mL (S-9+)	Rat liver S-9, PB/β-NF induced	+	+	Miller *et al.* (1995)

[1] 2,6-DAT dihydrochloride

[2] Positive only at the highest dose of 500 µg/ml

The genotoxicity of 2,6-DAT has been tested in rats and mice *in vivo* in several different assays (Table 19). 2,6-DAT did not induce mutations in the *in vivo* assays conducted in rats or mice. One DNA adduct study observed a weak positive adduct spot at an extended autoradiogram developing time (14 hours) in rat liver (Taningher *et al.*, 1995), while another study in the same rat strain did not detect 2,6-DAT adducts in the liver (La and Froines, 1993). DNA damage as measured by the Comet assay was detected in SD rat liver, but not stomach, following 2,6-DAT exposure for 29 days, although the positive control in this study failed to meet the study's acceptance criteria, rendering the validity of this finding uncertain (Rothfuss *et al*., 2010). DNA damage as measured by alkaline elution was reported in another study in albino SD rat liver (Allavena *et al*., 1992). Other DNA damage assays in rats and mice testing single and lower doses of 2,6-DAT were negative (Table 19). 2,6-DAT induced UDS in rat liver in one of three studies, MN in one study in rat bone marrow, but not in other rat studies in bone marrow, liver, or peripheral blood cells. 2,6-DAT inhibited DNA synthesis in mouse testes in a single study (Table 19).

Table 19. *In vivo* genotoxicity studies of 2,6-DAT

Endpoint	Species/ Strain/ Sex	Tissues Analyzed	Dose	Route	Results	Reference
Mutation	Big Blue® B6C3F1 transgenic mice, male	Liver DNA (for *lacI* mutations)	1000 ppm for 30 or 90 days	diet	-	Hayward *et al.* (1995)
	Muta® CD2-lacZ80/HazfBR transgenic mice, male	Skin at dose site, liver, and kidney DNA (for *LacZ* mutations)	12.5 mg/kg/day for 28 days	dermal	-	Kirkland and Beevers (2006)
	F344 *gpt* delta transgenic rats, male	Liver DNA (for *gpt* mutations)	500 ppm for 13 weeks	diet	-	Toyoda-Hokaiwado *et al.* (2010)
		Liver DNA (for Spi^- selection of deletions)			-	
		Kidney DNA (for *gpt* mutations)			-	
	F344/NSlc-Tg (*gpt* delta) transgenic rats, male	Liver genomic DNA (for *gpt* mutations)	60 mg/kg/day for 28 days	oral gavage	-	Sui *et al.* (2012)
DNA Adduct Formation	Fisher 344 rats, male	Liver	134.6 mg/kg, harvested after 18 h	*i.p.* injection	-	La and Froines (1993)
	Fisher 344 rats, male	Liver	250 or 500 mg/kg, harvested after 18 h	*i.p.* injection	+[1]	Taningher *et al.* (1995)
DNA Damage (Comet Assay or alkaline elution assay)	Albino SD rats, male	Hepatocytes	1000 mg/kg or 2000 mg/kg	oral gavage	+[2]	Allavena *et al.* (1992)
	ddY mice, male	Multiple tissues	60 mg/kg	oral gavage	-	Sasaki *et al.* (1999); Sekihashi *et al.* (2002)
	Wistar rats, male		250 mg/kg		-	
	SD rats, male	Liver DNA	15, 30, or 60 mg/kg/day for 29 days	oral gavage	+[3]	Rothfuss *et al.* (2010)
			125, 250, or 500 mg/kg/day for 3 days		-	
		Stomach DNA	15, 30, or 60 mg/kg/day for 29 days		-[3]	
			125, 250, or 500 mg/kg/day for 3 days		-	
UDS	Fisher 344 rats, male	Hepatocytes	150 mg/kg bodyweight in	oral gavage	-	Mirsalis *et al.* (1982)

Endpoint	Species/ Strain/ Sex	Tissues Analyzed	Dose	Route	Results	Reference
			corn oil			
	F344 or PVG rats (not specified)	Hepatocytes	300 mg/kg bodyweight	Oral gavage	-	George and Westmoreland (1991)
	Albino SD rats, male	Hepatocytes	1000 mg/kg or 2000 mg/kg	oral gavage	+[2,4]	Allavena *et al.* (1992)
MN	Fisher 344 rats, male and female	Bone marrow polychromatic erythrocytes	300 or 600 mg/kg (Ultrasonicated in water)	oral gavage	+	George and Westmoreland (1991)
	Albino SD rats, male	Hepatocytes	1000 mg/kg	oral gavage	-	Allavena *et al.* (1992)
		Bone marrow polychromatic erythrocytes	1000 mg/kg or 2000 mg/kg[3]		-	
	SD rats, male	Bone marrow polychromatic erythrocytes	15, 30, or 60 mg/kg/day for 29 days	oral gavage	-	Rothfuss *et al.* (2010)
			125, 250, or 500 mg/kg/day for 3 days		-	
	F344 *gpt* delta transgenic rats, male	Peripheral blood cells	500 ppm for 13 weeks	diet	-	Toyoda-Hokaiwado *et al.* (2010)
	Crl:CD (SD) rats, male	Liver hepatocytes or bone marrow erythrocytes	50 mg/kg/day for 5 days or 14 days; or 25, 50, or 100 mg/kg/day for 28 days	oral gavage	-	Takasawa *et al.* (2013)
Inhibition of DNA synthesis[5]	C57BL/6 × C3H mice, male	Testes	30-100 mg/kg	*i.p.* injection	+	Greene *et al.* (1981)

[1] The ^{32}P-postlabeling technique was used on the DNA adducts. The 500 mg/kg group had one weak positive adduct spot on the autoradiogram with extended autoradiogram exposure time (14 h).

[2] These two doses were given under two different protocols. See reference for details.

[3] The validity of the results is questionable, as the positive control failed to meet the predefined acceptance criteria. However, since a positive result was obtained in the liver, the deviation in the positive control was not considered to invalidate the results by the authors.

[4] Positive with the 2000 mg/kg dose and protocol.

[5] The authors stated that “most carcinogens bind to DNA and thereby have the capability of suppressing template activity”, and therefore “any genetically active material capable of reaching the testes might be expected to inhibit testicular DNA synthesis”.

3,4-DAT

The genotoxicity of 3,4-DAT has been investigated in three studies in *Salmonella typhimurium* and one *in vivo* study in mice.

3,4-DAT has been tested in the *Salmonella* reverse mutation assay using several different test strains, including TA98, 100, 1535, 1537, and 1538. Only one weakly positive result was reported, in TA1538 in the presence of rat liver microsomes induced with β-naphthoflavone (β-NF) (Table 20).

Table 20. Mutagenicity studies of 3,4-DAT in *Salmonella*

Strain	Concentration Tested	Activation System	Results		Reference
			-S-9	+S-9	
TA1538	10 µg/plate	Rat liver microsomes, β-NF induced or non-induced	NT	+/-[1]	Dybing and Thorgeirsson (1977)
		Mouse liver microsomes, β-NF induced or non-induced	NT	-	
TA98	Unspecified	Rat liver S-9, Aroclor 1254 induced or non-induced; or mouse liver S-9, non-induced	NT	-[2]	Greene *et al.* (1979)
TA100			NT	-[2]	
TA98	3 µmol/plate	Rat liver S-9, Aroclor 1254 induced	-	-	Florin *et al.* (1980)
TA100			-	-	
TA1535			-	-	
TA1537			-	-	

NT, not tested

[1] Weakly positive with β-NF induced microsomes

[2] Liver enzyme induction with 3,4-DAT before preparation of S-9 did not affect the mutagenicity

3,4-DAT was tested *in vivo* in a mouse assay for inhibition of DNA synthesis by Greene *et al.* (1981), and found to inhibit DNA synthesis in the testes (Table 21).

Table 21. *In vivo* genotoxicity studies of 3,4-DAT

Endpoint	Species/ Strain/Sex	Tissue Analyzed	Dose	Route	Results	Reference
Inhibition of DNA synthesis[1]	C57BL/6 × C3H mice, male	Testes	200-300 mg/kg	*i.p.* injection	+	Greene *et al.* (1981)

[1] The authors stated that "most carcinogens bind to DNA and thereby have the capability of suppressing template activity", and therefore "any genetically active material capable of reaching the testes might be expected to inhibit testicular DNA synthesis".

3.3.3 In Vitro Cell Transformation Studies

Cell transformation assays are designed to detect a change in growth pattern of cells that is indicative of loss of contact inhibition, a phenotype that is characteristic of cancer cells. The results of cell transformation assays often correlate with *in vivo* carcinogenicity in rodents (Heidelberger *et al.*, 1983; Creton *et al.*, 2012). The *in vitro* morphological transformation assay in Syrian Hamster Embryo (SHE) cells has been reported as a reliable system that identifies both genotoxic and non-genotoxic rodent carcinogens (Ahmadzai *et al.*, 2012; Benigni *et al.*, 2013; Benigni *et al.*, 2015). Bhas 42 cells, which are generated from BALB/3T3 cells through the transfection of a plasmid (pBR322 containing Ha-MuSV-DNA, clone H1 (v-Ha-*ras*)), can also be used in cell transformation studies. Using two different seeding density and treatment regimens, the Bhas 42 cell transformation assay has the ability to differentiate tumor initiators from promoters (Sakai *et al.*, 2010).

2,4-DAT, 2,5-DAT, 2,6-DAT, and 3,4-DAT have been tested for the ability to transform SHE cells, and 2,3-DAT, 2,4-DAT, 2,5-DAT, 2,6-DAT and 3,4-DAT have been tested for the ability to transform Bhas 42 cells. These studies are briefly discussed in this section and summarized in Table 22 and 23 below.

2,4-DAT has tested positive in 11 out of 12 *in vitro* cell transformation studies conducted with SHE cells (Table 22). The potency of transformation varied with experimental conditions, such as length of exposure, pH, feeder cells, and plating efficiency. In one study, the lowest active concentration was 0.1 µg/mL (Maire *et al.*, 2012), whereas in other studies 2,4-DAT was not active at levels below 40 µg/mL (Kerckaert *et al.*, 1998).

2,4-DAT was also tested in the Bhas 42 cell transformation system, and shown to be positive in the 'tumor initiation' cell transformation assay, but not the 'tumor promotion' assay (Sakai *et al.*, 2010).

Table 22. *In vitro* Cell Transformation Studies of 2,4-DAT

Assay System	Concentration	Results	Reference
Secondary Golden Syrian hamster embryo (SHE) cells	Up to 50 µg/mL for 8 days	+	Pienta *et al.* (1977)
Secondary SHE cells	Up to 50 µg/mL for 6 days	+[1]	Greene and Friedman (1980)
SHE cells	Up to 30 µg/mL	-	Holen *et al.* (1990)
SHE cells (pH 6.7)	Up to 65 µg/mL for 7 days	+	LeBoeuf *et al.* (1996); Kerckaert *et al.* (1998)
SHE cells	70 µg/mL	+	Engelhardt *et al.* (2004)
SHE cells	50-1,250 µg/mL for 24 hours	+	Pant *et al.* (2004)
SHE cells (pH 6.7)	Up to 65 µg/mL for 7 days	+	Pant *et al.* (2008)
SHE cells (no feeder cells)	Up to 80 µg/mL for 7 days	+	
SHE cells	50 µg/mL	+	Ahmadzai *et al.* (2012)
SHE cells (pH 7.0)	0.1 to 100 µg/ml for 7 days[2]	+[3]	Maire *et al.* (2012)
SHE cells (pH 6.7)	Up to 100 µg/mL[4]	+	Pant *et al.* (2012)
SHE cells (pH 6.7)	Up to 80 µg/mL for 7 days	+	Plottner *et al.* (2013)
Bhas 42 cells initiation assay	Up to 50 µg/mL	+	Sakai *et al.* (2010)
Bhas 42 cells promotion assay	Up to 20 µg/mL	-	

[1] Positive in three out of six experiments (including two high sensitivity experiments and one low sensitivity experiment). The sensitivity is defined by the activity of the positive control. 2,4-DAT is considered positive because it was positive in a low sensitivity experiment.
[2] Assays were performed by four independent laboratories, with a common dose range of 5-40 µg/ml.
[3] Positive in all four assays performed at different laboratories.
[4] Assays were performed by three independent laboratories, with a common dose range of 10-70 µg/ml.

2,5-DAT, 2,6-DAT, and 3,4-DAT were tested for the ability to transform secondary SHE cells. In studies by Greene and Friedman (1980), 2.5-DAT, 2,6-DAT, and 3.4-DAT were weakly positive in 2/5, 3/6, and 2/5 experiments, respectively. They were considered as weakly positive because these isomers tested positive only in the highly sensitive experiments, but negative in the low sensitive experiments (Greene and Friedman,

1980). Other studies of 2,6-DAT in SHE cells were negative (LeBoeuf *et al.*, 1996; Kerckaert *et al.*, 1998; Engelhardt *et al.*, 2004; Pant *et al.*, 2008).

In the Bhas 42 cell transformation assay, 2,3-DAT, 2,5-DAT, and 3,4-DAT were positive in the 'tumor promotion' assay (Sakai *et al.*, 2010). 2,6-DAT was negative in both the initiation and promotion assays in the Bhas 42 cell transformation system (Sakai *et al.*, 2010).

Table 23. *In vitro* Cell Transformation Studies of 2,3-, 2,5-, 2,6-, and 3,4-DAT

DAT	Assay System	Concentration	Results	Reference
2,3-DAT	Bhas 42 cells initiation assay	Up to 50 µg/mL	-	Sakai *et al.* (2010)
	Bhas 42 cells promotion assay	Up to 100 µg/mL	+	
2,5-DAT	Secondary SHE cells	Up to 10 µg/mL for 6 days	weakly +[1]	Greene and Friedman (1980)
	Bhas 42 cells initiation assay[2]	Up to 13 µg/mL	-	Sakai *et al.* (2010)
	Bhas 42 cells promotion assay[2]	Up to 40 µg/mL	+	
2,6-DAT	Secondary SHE cells	Up to 200 µg/mL for 6 days	weakly +[1]	Greene and Friedman (1980)
	SHE cells (pH 6.7)	Up to 2000 µg/mL for 24 hr,	-	LeBoeuf *et al.* (1996); Kerckaert *et al.* (1998)
		Up to 225 µg/mL for 7 days	-	
	SHE cells	150 µg/mL	-	Engelhardt *et al.* (2004)
	SHE cells (pH 6.7)	Up to 225 µg/mL for 7 days	-	Pant *et al.* (2008)
	SHE cells (without feeder cells)	Up to 200 µg/mL for 7 days	-	
	Bhas 42 cells initiation assay	Up to 300 µg/mL	-	Sakai *et al.* (2010)
	Bhas 42 cells promotion assay	Up to 200 µg/mL	-	
3,4-DAT	Secondary SHE cells	Up to 50 µg/mL for 6 days	weakly +[1]	Greene and Friedman (1980)
	Bhas 42 cells initiation assay	Up to 20 µg/mL	-	Sakai *et al.* (2010)
	Bhas 42 cells promotion assay	Up to 30 µg/mL	+	

[1] weakly +, weakly positive.

[2] 2,5-DAT dihydrochloride was the test substance in these assays.

3.3.4 Animal Tumor Pathology

The pathology of tumor sites observed in the animal cancer bioassays of 2,5-DAT sulfate and 2,6-DAT dihydrochloride are discussed below. Pathology of the tumor sites observed in bioassays of 2,4-DAT (see Table 2) are not discussed here, since 2,4-DAT is already listed individually as a carcinogen under Proposition 65.

2,5-DAT

Testicular interstitial cell tumors

Testicular interstitial cell tumors, also referred to as Leydig cell tumors, were increased in 2,5-DAT sulfate-treated male F344 rats, as compared to controls (NCI, 1978). Interstitial cells are located in the interstitium of the testis, between the seminiferous tubules. There is a continuum of interstitial cell proliferative response, ranging from hyperplasia to adenomas and carcinomas (Boorman *et al*., 1990). Differential diagnosis is based on size of the lesion. Interstitial cell adenomas and carcinomas are aggregated for carcinogen identification (IARC, 2006; McConnell *et al*., 1986).

Interstitial cell tumors are known to occur with high spontaneous incidence in untreated aged Fischer rats. While the NCI report does not include the historical data on incidence of these tumors at the laboratory where the study was conducted, Goodman *et al.* (1979) reported the spontaneous incidence as 80.5 percent in untreated controls in NCI Carcinogenesis Testing Program studies (carried out by contract laboratories) from 1972-1978. Boorman *et al.* (1990) reported a spontaneous incidence of 83 percent testicular interstitial adenoma in male F344 rats at 24 months old. The incidences observed in the low- and high-dose controls in the NCI 1978 study, 73% and 79%, respectively, are generally consistent with these other reports.

Lung tumors

Lung tumors, namely alveolar/bronchiolar adenomas and carcinomas, were increased in female B6C3F1 mice treated with 2,5-DAT sulfate, as compared to controls (NCI, 1978). Mouse lung adenomas have the potential to progress to malignant carcinomas and these tumors are aggregated for carcinogen identification (McConnell *et al.*, 1986; IARC, 2006).

2,6-DAT

Liver tumors

Hepatocellular adenomas and carcinomas combined were increased in male F344 rats and hepatocellular carcinomas were increased in female B6C3F1 mice treated with 2,6-DAT dihydrochloride (NCI, 1980).

The liver tumors observed in the male rat study were referred to as hepatocellular neoplastic nodules and hepatocellular carcinomas (NCI, 1980). Neoplastic nodule is an

older term used for hepatocellular adenoma, although now the term hepatocellular adenoma is preferred (Bannasch and Zerban, 1990). Hepatocellular carcinomas are rare in untreated male rats, with an incidence of 0.7 percent reported in NTP feeding studies conducted up to 1997 (Haseman *et al.*, 1998). The incidence of hepatocellular carcinomas observed in 2,6-DAT dihydrochloride-treated male rats in the 1980 NCI study was 4.3 percent in the low-dose group and 2.4 percent in the high-dose group. Hepatocellular adenomas and carcinomas arise from the same cell type, and adenomas can progress to carcinomas. For this reason, these two tumors phenotypes are aggregated when evaluating study results (McConnell *et al.*, 1986; IARC, 2006).

Three hepatocellular carcinomas were observed in 2,6-DAT dihydrochloride-treated female mice in the high-dose group (incidence 6.7%), with none observed in the control or low-dose group (NCI, 1980). Hepatocellular adenomas were observed in the control, low-, and high-dose groups, with no discernable dose-response. Hepatocellular carcinomas were first observed at week 93, while hepatocellular adenomas were not observed until week 105. Hepatocellular carcinomas can have variable growth patterns such as trabecular, glandular, or solid, with varying degrees of differentiation. No further description was reported by the study authors (NCI, 1980). Hepatocellular carcinomas in untreated female B6C3F1 mice were reported to occur with an incidence of 8.4 percent (113/1350, range: 0-20 percent) in NTP feeding studies conducted up to 1997 (Haseman *et al.*, 1998).

Pancreatic tumors

Pancreatic islet cell adenomas were increased in male F344 rats treated with 2,6-DAT dihydrochloride (9.3% incidence in the high-dose group) (NCI, 1980). The pancreas is comprised of the exocrine and endocrine pancreas. The endocrine pancreas is made up of the islets of Langerhans (i.e., the islets), which are collections of cells distributed throughout the pancreas and comprise about 1-2 percent of the total pancreatic tissue. Pancreatic islet cell adenomas are reported to occur in untreated male F344 rats with an incidence of 4.0 percent in NTP feeding studies up to 1997 (Haseman *et al.*, 1998). Pancreatic islet cell adenomas and carcinomas arise from the same cell type, and adenomas can progress to carcinomas (McConnell *et al*., 1986).

3.3.5 Effects on Cell Proliferation

Cunningham *et al.* (1991) investigated the effects of 2,4-DAT and 2,6-DAT dihydrochloride on cell proliferation in male rat liver (a target tumor site for each isomer) in male F344 rats at dose levels comparable to the 1979 and 1980 NCI bioassays on these isomers. The rats received daily gavage doses of 12.5 or 25 mg/kg/day 2,4-DAT,

or 25 or 50 mg/kg/day 2,6-DAT dihydrochloride for nine days, and then were sacrificed. As shown in Table 24, 2,4-DAT induced a dose-dependent increase in liver cell proliferation, but 2,6-DAT dihydrochloride did not.

Table 24. Effects on cell proliferation in rat liver

	Vehicle Control	2,6-DAT		2,4-DAT		Positive Control
Dose	0.01 M HCl	25 mg/kg/day	50 mg/kg/day	12.5 mg/kg/day	25 mg/kg/day	0.4 mL CCl_4 in corn oil
Hepatocyte proliferation[1]	1	0.6	0.6	11.4*	18.5*	45.5*

[1] Fold increase over vehicle control. Proliferation is quantified as the percentage of bromodeoxyuridine (BrdU) positive cells (>1000 hepatocytes counted).

* Significantly different from vehicle control at $p < 0.001$: $n = 5$.

Pons *et al.* (1999) examined the effect of 2,6-DAT and 2,4-DAT on the viability and growth of human lung fibroblast cells by a tetrazolium-based cell viability assay. The studies were performed with two cell densities (2,500 or 5,000 cells/well in a 96-well plate) and different concentrations of chemicals, and treatment lasted either 24 or 48 hours. 2,6-DAT exhibited both a cytotoxic and proliferative effect, depending on the concentration, cell density, and the time of treatment. 2,6-DAT stimulated cell growth in the low cell density wells at the highest concentration (10^{-4} mol/L) after 48 h treatment, and inhibited cell growth in the high cell density wells at lower concentrations (10^{-7} to 10^{-6} mol/L) after 24 h treatment. In contrast, 2,4-DAT decreased cell viability at multiple concentrations and in both high and low cell densities after 48 h treatment. After 24 h treatment the cytotoxicity of 2,4-DAT was less pronounced.

3.3.6 Effects on Gene/Protein Expression

3.3.6.1 Effects on gene expression

The Comparative Toxicogenomics Database (CTD) is a publically available database that provides information on chemical-gene/protein interactions, chemical-disease, and gene-disease relationships. Searches of the CTD for toxicogenomic data on each of the DAT isomers (except 2,4-DAT, which is already listed individually under Proposition 65) identified two gene expression studies, Nakayama *et al.* (2006) and Aeby *et al.* (2004).

The *in vivo* toxicogenomic studies by Nakayama *et al.* (2006) investigated the effect of 2,4-DAT and 2,6-DAT on gene expression in rat liver. Male Fischer rats received daily

oral doses of either 10 mg/kg/day 2,4-DAT or 10 mg/kg/day 2,6-DAT for 28 days, at which time liver gene expression was assessed. Gene expression analysis was performed by oligo microarrays consisting of 6709 unique genes and control genes. Results were presented for 54 genes. The data are expressed as the ratios of the levels in treated rats relative to the levels in time-matched vehicle controls. The effect of 2,4-DAT treatment on rat liver gene expression in this study was generally greater than that of 2,6-DAT. Table 25 summarizes expression levels for genes related to cell cycle and apoptosis. The effect on cell cycle observed in this microarray study is consistent with findings in the study by Gulati *et al.* (1989) in CHO cells, where exposure to 2,6-DAT dihydrochloride (at doses ranging from 250-1500 µg/ml) resulted in marked cell cycle delay. For more data on other genes examined in this study, see Appendix B.

Table 25. Expression of cell cycle and apoptosis related genes in livers of rats treated with 2,6-DAT and 2,4-DAT (Nakayama *et al*., 2006)

Biological Process	Genes[1]	Description	Normalized Ratio[2]	
			2,6-DAT	2,4-DAT
Cell cycle	*Ccng1* [BI280715]	Cyclin G1	1.4	2.69
	Ccng1 [AI009504]		1.18	2.23
	Cdkn1a	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.24	2.1
Apoptosis	*Bax*	Bcl2-associated X protein	1.08	1.39
	Tnfrsf6 [AI231531]	Tumor necrosis factor receptor superfamily, member 6	0.86	1.81
	Tnfrsf6 [CV796105]		0.86	1.28
	Wig1	Wild-type p53-induced gene 1	1.04	1.14
	C7	Complement component 7	0.78	1.16

[1] For genes that were identified with multiple oligos, the GenBank ID is shown in the brackets for each oligo.
[2] The average of four replicates is shown here, as the normalized ratio of signal intensity from treated animals to that from time-matched controls.

In an *in vitro* study by Rohrbeck *et al.* (2010), exposure of Balb/c 3T3 cells to 50 µM 2,4-DAT resulted in a 6.68 fold increase in Cyp1a1 gene expression. In another *in vitro* study by Aeby *et al.* (2004), human monocytes from peripheral blood were cultured in the presence of interleukin-4 (IL-4) and granulocyte macrophage colony-stimulating factor (GM-CSF) to induce the dendritic cell (DC) phenotype. In these immature DC-like cells, incubation with 2,5-DAT (57-454 µM) resulted in decreased expression of Aquaporin 3 (AQP3) mRNA and increased expression of Interleukin-1B (IL-1B) mRNA. The protein products of both of these genes are involved in DC activation. AQP3 belongs to the Aquaporin family of membrane channel proteins and is responsible for transepithelial transport of liquids, such as water and glycerol (Tradtrantip *et al.*, 2009). Aquaglycerolproteins such as AQP3 and other aquaglycerol proteins are highly expressed in tumor tissue compared to normal tissues, and are involved in cell migration, proliferation, and angiogenesis (Verkman *et al.*, 2008). The cytokine IL-1B is

secreted by activated DCs. It is highly expressed in tumor tissues (Apte *et al.*, 2006), and is thought to be important in early tumor angiogenesis (Carmi *et al.*, 2013).

3.3.6.2 Effect on CYP1A protein expression

In the study by Cheung *et al.* (1996), groups of male Wistar albino rats received single *i.p.* injections of 2,3-, 2,4-, 2,5-, or 2,6-DAT in corn oil, at 3 dose levels (10, 20, or 40 mg/kg). Control animals received corresponding volumes of corn oil. Immunoblot of the hepatic microsomal proteins from animals exposed to 2,3-DAT showed a dose-dependent increase in protein expression of CYP1A1 and CYP1A2. In the vehicle control, only CYP1A2 was detected; CYP1A1 was not detected. The authors used anti-CYP1A1 to detect both CYP1A1 and CYP1A2, and in the case of 2,4-, 2,5-, and 2,6-DAT, the two bands were too close to be distinguishable. 2,4-DAT increased the expression of CYP1A, but the increase was not strong. Exposure to 2,5-DAT or 2,6-DAT had no effect on CYP1A protein expression. A competitive receptor binding assay in the same study found that both 2,3-DAT and 2,4-DAT displaced ^{3}H-radiolabeled TCDD from rat hepatic Aryl Hydrocarbone Receptor (AhR), which transcriptionally regulates the expression of CYP1A1 and CYP1A2 (Cheung *et al.*, 1996).

CYP1A enzymes carry out the activation of planar carcinogens through reactions such as N-hydroxylation (Guengerich and Shimada, 1991; Go *et al.*, 2015).

3.3.7 ToxCast High-throughput in vitro Assays (2,3-DAT, 2,4-DAT and 3,4-DAT)

ToxCast™ is a chemical prioritization research program developed by US EPA (Dix *et al.*, 2007). It is a multi-year effort launched in 2007 that utilizes various *in vitro* systems to identify chemical activity in a battery of high-throughput screening (HTS) assays. As of 2015, there are 1860 chemicals and 821 *in vitro* assay endpoints in the ToxCast database.

OEHHA has searched the ToxCast database via the Interactive Chemical Safety for Sustainability (iCSS) Dashboard (http://actor.epa.gov/dashboard/), and located chemical activity data on three DAT isomers: 2,3-DAT, 2,4-DAT, and 3,4-DAT (Table 26) (accessed on 4/17/2015). Lists of the ToxCast HTS assays for which each of these isomers is active are provided in Appendix C.

Table 26. Summary of ToxCast HTS assay activity for 2,3-, 2,4-, and 3,4-DAT

Isomer	2,3-DAT	2,4-DAT	3,4-DAT
Number of active assays /tested assays	70/421	7/392	49/405
Range of AC_{50} values[1] (µM)	0.000339-164	4.28-32.2	0.68-89.1

[1] The AC_{50} indicates the chemical concentration that induces a half-maximal assay response.

This section highlights the ToxCast HTS assays in which 2,4-DAT and at least one other DAT isomer were active. As shown in Table 27, there are four assays in which all three DATs were active, one assay in which both 2,3-DAT and 2,4-DAT were active, and one assay in which both 2,4-DAT and 3,4-DAT were active. In addition, there are 26 assays in which both 2,3-DAT and 3,4-DAT were active, but in which 2,4-DAT was inactive (summarized in Table 28).

Table 27. ToxCast HTS Assays[1] in which 2,4-DAT and at least one other DAT isomer are active

Assays	Assay Description	AC_{50} (μM)		
		2,3-DAT	2,4-DAT	3,4-DAT
Tox21_AhR (cell-based)	Luciferase reporter assay measuring aryl hydrocarbon receptor (AhR) transcription factor activity in HepG2 (human hepatocellular carcinoma) cells.	48.9	4.28	17.8
ATG_Ahr_CIS_up (cell-based)	Measurement of inducible changes in transcription factor activity as indicated by changes in fluorescence intensity signals. The level of fluorescent reporter indicating activity at the cis-acting AhR response element is quantified in HepG2 cells.	0.978	4.55	25.9
BSK_CASM3C_Throm-bomodulin_up (cell-based)	ELISA immunoassay measuring Thrombomodulin (TM) expression in primary culture of human vascular smooth muscle cells grown in IL-1β, TNF-α, and IFN-γ stimulated environment.[1]	11.2	7.48	10
BSK_hDFCGF_Prolifera-tion_down (cell-based)	Sulforhodamine protein staining indicating cell proliferation in primary culture of human skin fibroblasts grown in IL-1β, TNF-α, IFN-γ, EGF, bFGF, and PDGF-BB stimulated environment.[2]	10.3	15.2	13
NVS_ENZ_hPTPRC (cell-free)	Cell-free assay measuring enzyme inhibition of human protein tyrosine phosphatase, receptor type C (PTPRC).	21.5	32.2	inactive
NVS_ENZ_rabI2C (cell-free)	Cell-free assay measuring enzyme inhibition of creatine kinase (CKB) derived from rabbit brain.	inactive	20.8	8.98

[1] As of 4/17/2015

[2] Acronyms for growth factors: IL-1β, interleukin 1 beta; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; EGF, epithelial growth factor; bFGF, basic fibroblast growth factor; PDGF-BB, platelet-derived growth factor BB

The four cell-based assays in which 2,3-DAT, 2,4-DAT, and 3,4-DAT were all active (the first four rows in Table 27) shed some light on common activities of these three isomers.

All three DATs induced aryl hydrocarbon receptor (AhR) transcriptional activity, as shown by two independent assays evaluating this molecular target. Both assays measured reporter gene expression induced by the binding of activated AhR to the AhR response element. Activated AhR leads to transcription of downstream genes, such as the CYP1A1 and CYP1A2 enzymes (Go *et al.*, 2015). Since CYP1A enzymes are

responsible for the *N*-hydroxylation of DATs (Cheung *et al.*, 1996), the induction of AhR transcriptional activity by the DATs leads to the chemicals' own metabolic activation.

The thrombomodulin (TM) assay results showed that these DATs induce protein expression of TM, a thrombin receptor in endothelial cell membranes that is involved in reducing blood coagulation. Altered TM expression levels have been reported in some cancers. An increase in TM expression was linked with increased invasiveness in mouse breast tumor cells (Niimi *et al.*, 2005). Other studies have linked decreased expression of TM to increased invasiveness and poorer prognosis of colorectal (Hanly *et al.*, 2006) and non-small cell lung cancers (Liu *et al.*, 2010). Decreased TM expression has also been observed in prostate cancer cells (Menschikowski *et al.*, 2012).

All three DATs were found to decrease cell proliferation in the ToxCast HTS assay conducted in fibroblasts.

2,3-DAT and 2,4-DAT were both active in an assay evaluating inhibition of human protein tyrosine phosphatase, receptor type C (PTPRC, also known as CD45), a transmembrane receptor-like tyrosine phosphatase that is expressed exclusively in hematopoietic cells, and is involved in B- and T-cell receptor signaling (Andersen *et al.*, 2004). PTPRC/CD45 expression sensitizes hematopoietic progenitor cells to C-X-C motif chemokine 12 (CXCL12)-induced motility by regulating SRC kinase activity; the activity of PTPRC/CD45 is inhibited by the BCR-ABL oncogenic fusion protein (Williamson *et al.*, 2013). PTPRC/CD45 has been recognized as a tumor suppressor in T-cell acute lymphoblastic leukemia, as its loss-of-function mutation activates the Janus kinase (JAK) family kinases, and its down-regulation induces downstream signaling of these oncoproteins (Porcu *et al.*, 2012). The inhibition effect of 2,3- and 2,4-DAT on PTPRC/CD45 indicates potential activation of oncoproteins.

2,4-DAT and 3,4-DAT were both active in an assay examining inhibition of creatine kinase derived from rabbit brain (CKB). CKB catalyzes the reversible transfer of the N-phosphoryl group from phosphocreatine to ADP to generate ATP and plays a role in cellular energy homeostasis (Zhang *et al.*, 2009). The expression of CKB has been shown to be down-regulated in various human cancer tissues. The activity of CKB in cervical cancer tissues is lower than in normal tissues (Choi *et al.*, 2001). In human gastric and colonic adenocarcinoma, brain-type creatine kinase was found to be downregulated, whereas ubiquitous mitochondrial creatine kinase was upregulated (Patra *et al.*, 2008). In human colon tumors, subcellular levels of CKB were lower than in the matched adjacent normal tissues (Balasubramani *et al.*, 2006). The expression of CKB is progressively decreased upon transformation of mouse skeletal muscle into

sarcoma, making CKB a potential diagnostic marker for malignancy of the tissue (Patra *et al.*, 2008). On the other hand, in ovarian cancer cells, CKB knock-down inhibited cancer cell proliferation and induced apoptosis (Li *et al.*, 2013). The inhibition of CKB activity by 2,4-DAT and 3,4-DAT suggests these two DAT isomers could affect cell energy homeostasis.

Of the 26 assays in which both 2,3-DAT and 3,4-DAT (the two *ortho*-DAT isomers) were active, several indicate upregulation of various transcription factor activities, others indicate modulation of protein expression (either upregulation or downregulation) or function, and some indicate effects on cell proliferation or growth. The active assays are summarized in Table 28.

Table 28. ToxCast HTS assays in which 2,3-DAT and 3,4-DAT are active

Endpoints	Molecular Targets	No. of Assays
Upregulation of transcription factor activities	Peroxisome proliferator-activated receptors (PPARα, PPARδ, or PPARγ)[1]	1
	The activator protein 1 (AP-1), activation of the proto-oncogene c-Jun signaling pathway	1
	Early growth response 1 (EGR1)	1
	Nuclear factor, erythroid 2-like 2 (Nrf2)	2
	Vitamin D receptor (VDR)	1
	POU class 2 homeobox 1 (Pou2f1)	1
	metal-regulatory transcription factor 1 (Mrf1)	1
	RAR-related orphan receptors (RORA, RORB, or RORC)[1]	1
Upregulation of protein expression	Thrombomodulin (TM)	1
	Interleukin-8 (IL-8)	1
Downregulation of protein expression	Vascular cell adhesion molecule 1 (VCAM1)	4
	HLA class II histocompatibility antigen, DR alpha chain (HLA-DRA)	1
	C-C motif chemokine 26 (CCL26), inhibition of the histamine and interleukin-4 (IL-4) pathway	1
	Colony stimulating factor 1 (CSF-1)	2
	Matrix metallopeptidase 9 (MMP9)	1
	Prostaglandin E receptor 2 (subtype EP2) (Ptger2)	1
	Downregulation of Selectin E protein expression	1
Modulation of protein function	Induction of transporter function of human solute carrier family 6 (neurotransmitter transporter), member 2 (SLC6A2)	1
Inhibition of cell proliferation or growth	Not identified	2

[1] The assay does not differentiate the subtype of these receptors

3.3.8 Structure Activity Comparisons

3.3.8.1 Comparison among the DAT isomers

As described in Section 3.3.2, all DATs except for 3,5-DAT were tested for genotoxicity. The activities have been summarized briefly below and in Table 29.

- Mutagenicity
    - In *Salmonella*, evidence of mutagenicity was observed for 2,3-DAT, 2,4-DAT, 2,5-DAT, 2,6-DAT, and 3,4-DAT[14].
    - *In vitro* mutagenicity in mammalian cells was observed for 2,4-DAT and 2,6-DAT.
    - *In vivo* mutagenicity was observed in *Drosophila*, mice, and rats for 2,4-DAT.

- Chromosomal effects
    - In yeast, intrachromosomal recombination was observed for 2,4-DAT and 2,6-DAT.
    - In mammalian cells *in vitro*, chromosomal effects were observed for 2,4-DAT (CA, MN, SCEs), 2,5-DAT (CA), and 2,6-DAT (CA, MN, SCEs).
    - *In vivo*, chromosomal effects were observed for 2,4-DAT (MN, SCEs) and 2,6-DAT (MN).

- DNA damage and other effects
    - In *Salmonella*, evidence of DNA damage was observed for 2,4-DAT, 2,5-DAT, and 2,6-DAT.
    - In mammalian cells *in vitro*, the following was observed
        - 2,4-DAT: DNA damage, DNA covalent binding and adduct formation, and UDS
        - 2,5-DAT: DNA damage, UDS
        - 2,6-DAT: DNA covalent binding, UDS
    - *In vivo*, the following was observed
        - 2,4-DAT: DNA damage, DNA adduct formation, UDS and inhibition of DNA synthesis
        - 2,5-DAT: DNA damage, inhibition of DNA synthesis
        - 2,6-DAT: DNA damage, DNA adduct formation, UDS, and inhibition of DNA synthesis
        - 3,4-DAT: Inhibition of DNA synthesis

All DATs but 3,5-DAT were tested for *in vitro* transformation, and they were all positive.

Tumor findings from carcinogenesis bioassays conducted in animals with the DATs were observed with the 2,4-, 2,5-, and 2,6-DAT. No information for other isomers was identified. More details on the tumor findings are presented in Table 29.

[14] 3,4-DAT was weakly positive in one study in *Salmonella*

3.3.8.2 Comparison with other structurally-related chemicals

The DATs are monocyclic primary aromatic diamines with a methyl group attached to the benzene ring. OEHHA used Chemotyper (https://chemotyper.org/), a tool for searching and highlighting chemotypes (chemical structures or subgraphs), to identify chemicals that share structural similarities with the DATs.

Chemotyper includes a public set of chemotypes named ToxPrint, which was developed by Altamira LLC for the US FDA Center for Food Safety and Applied Nutrition (CFSAN)'s Chemical Evaluation and Risk Estimation System (CERES) project. ToxPrint contains three basic subsets: generic structural fragments, Ashby-Tennant genotoxic carcinogen rules (Ashby and Tennant, 1988), and cancer threshold of toxicological concern (TTC) categories (Kroes *et al.*, 2004). ToxPrint identified the same seven unique chemotypes present in each of the DATs. These are:

- Bond: generic aromatic amine
- Bond: aromatic primary amine
- Bond: generic primary amine
- Chain: aromatic alkane, acyclic connection
- Chain: aromatic alkane, acyclic connection, no double bond
- Chain: aromatic alkane, generic
- Ring: benzene ring

Using these tools (Chemotyper and ToxPrint), OEHHA identified 42 chemicals among the 8000+ chemicals in the Tox21 chemical inventory (Collins *et al.*, 2008; Tox21, 2012) that fulfill the following rules:

- Contain all of these chemotypes
- Do not contain halogen atoms (in the free base form)
- Are monocyclic (one benzene ring)

Among these 42 chemicals, four are listed under Proposition 65 as carcinogens:

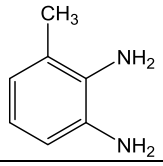
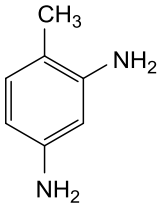
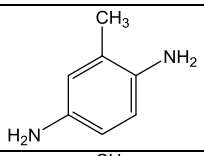
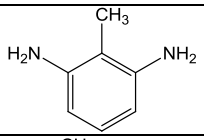
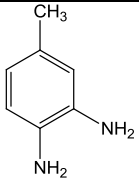
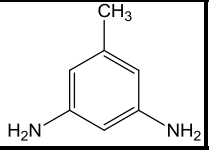
- *p*-Cresidine
- 2,6-Xylidine (2,6-dimethylaniline)
- *o*-Toluidine
- *o*-Toluidine hydrochloride

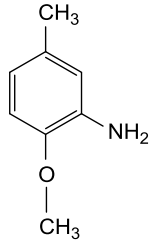
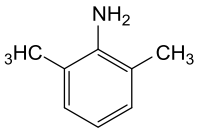
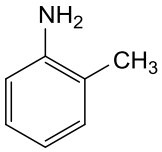
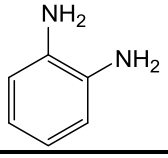
p-Cresidine, 2,6-xylidine, and o-toluidine were selected for the purpose of structure-activity comparison with the DATs, along with the carcinogenic aromatic diamine *o*-phenylenediamine, which lacks the aromatic alkane-related chemotypes. Each of the four comparison compounds is genotoxic (Sasaki *et al.*, 1998; WHO, 1998; Skipper *et al.*, 2010; Murata and Kawanishi, 2011; Chao *et al.*, 2012).

As a class, monocyclic amines undergo extensive N-hydroxylation by the cytochrome P450 enzymes *in vivo* to form reactive intermediates (McCarthy *et al.*, 1985; Skipper *et al.*, 2010). Specifically, 2,6-xylidine has been shown to undergo N-hydroxylation to form nitrenium ion intermediates and eventually DNA adducts (Chao *et al.*, 2012). One study in rats with ^{14}C-labeled *o*-toluidine showed that there was N-hydroxylation of the amino group (Son *et al.*, 1980), and another study measured N-hydroxy-*o*-toluidine in the urine of *o*-toluidine treated rats (Kulkarni *et al.*, 1983).

Information on the genotoxicity, *in vitro* cell transformation activity, and findings from animal cancer bioassays for the DATs and each of the four comparison chemicals is summarized in Table 29.

Table 29. Structure activity comparison between DATs and four structurally related monocyclic primary aromatic amines

Chemical	Structure	Genotoxicity[1]			*In vitro* cell transformation	Tumors in animal bioassays	Cancer Classification	
		Mutagenicity	Chromosomal effects	DNA damage and other effects			Proposition 65 (year)	IARC (year)
2,3-DAT		+	-	NT	+	NT	Currently under evaluation[2]	NE
2,4-DAT		+	+	+	+	Liver: male & female rats & female mice Vascular: male & female mice Lung: male mice Mammary: male & female rats Subcutaneous fibromas and sarcomas: male & female rats Bone: female rats Lymphoma: female mice	Listed[2] (1988)	2B (1987)
2,5-DAT		+	+	+	+	Lung: female mice Testicular: male rats	Currently under evaluation[2]	3 (1987)
2,6-DAT		+	+	+	+[3]	Liver: male rats & female mice Pancreatic islet cell: male rats	Currently under evaluation[2]	NE
3,4-DAT		+/-[3]	NT	+[4]	+	NT	Currently under evaluation[2]	NE
3,5-DAT		NT	NT	NT	NT	NT	Currently under evaluation[2]	NE

Chemical	Structure	Genotoxicity[1]			*In vitro* cell transformation	Tumors in animal bioassays	Cancer Classification	
		Mutagenicity	Chromosomal effects	DNA damage and other effects			Proposition 65 (year)	IARC (year)
p-Cresidine		+	+	+	NT	Liver: male rats (benign) & female mice Nasal: male & female rats Bladder: male & female rats & mice (NTP, 2014c)	Listed (1988)	2B (1987)
2,6-Xylidine		-	+	+	+	Liver: female rats Subcutaneous: male & female rats Nasal: male & female rats (NTP, 1990)	Listed (1991)	2B (1993)
o-Toluidine		+	+	+	+	Liver: female mice Vascular: male & female mice Connective tissue sarcoma: male & female rats Subcutaneous: male rats Mesothelium: male rats Bladder: male & female rats (NTP, 2014b)	Listed (1988)	1 (2012)
o-Phenylene-diamine		+	+	+	+	Liver: male rats & male & female mice (Weisburger *et al.*, 1978)	Listed (1998)	NE

NT: Not tested; NE: Not evaluated

[1] The references for the genotoxicity and *in vitro* transformation of chemicals other than the DATs are summarized here. For *p*-cresidine: Zeiger *et al.* (1988), Stoll *et al.* (2006), Sasaki *et al.* (1998), and JETOC (2005), as cited by CCRIS (2010)]; For 2,6-xylidine: NTP (1990), Galloway *et al.* (1987), Sasaki *et al.* (1999) and Matthews *et al.* (1993); For *o*-toluidine: NTP (2014b) and Danford (1991); For *o*-phenylenediamine: Garner and Nutman (1977), Sofuni *et al.* (1990), Gichner *et al.* (2001), and Cebulska-Wasilewska *et al.* (1998), and DHEW/NCI (1981), as cited by HSDB (2013).

[2] The listing of "diaminotoluene (mixed)" is currently under reconsideration

[3] Weakly positive in one study

[4] Positive in one assay (DNA synthesis Inhibition)

QSAR predictions

Quantitative structure activity relationship (QSAR) models predict the toxicity of chemicals by correlating physicochemical properties of related compounds to their biological activity quantitatively. Numerous QSAR models have been developed. Several QSAR models were used by OEHHA to predict the carcinogenicity of 2,3-, 2,5-, 2,6-, 3,4-, and 3,5-DAT. QSAR predictions were not made for the isomer 2,4-DAT because it is individually listed as a carcinogen on Proposition 65 and is included in the training sets of all QSAR models used in this document. Mutagenicity predictions for the five DAT isomers were also performed. The OECD (Organisation for Economic Co-operation and Development) and ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) guidelines were used to select QSAR models to generate predictions for the five DAT isomers.

The models chosen for the analysis were the following:

- VEGA (a platform developed through a collaboration between US EPA and a number of institutions from Europe under the sponsorship of ANTARES Project, CALEIDOS Project, CAESAR Project, Ministero della Salute, and ORCHESTRA Project) that utilized the following models for prediction and applicability domain analysis:
    - Carcinogenicity model (CAESAR) (version 2.1.8)
    - Benigni-Bossa Carcinogenicity (TOXTREE) (version 1.0.0-DEV)
    - Mutagenicity model (CAESAR) (version 2.1.12)
    - Mutagenicity SarPy model (version 1.0.6-DEV)
    - Benigni-Bossa Mutagenicity (TOXTREE) (version 1.0.0-DEV)
- Lazar, which was developed by a Swiss company (In silico toxicology GMBH, 2011) for predicting toxicological endpoints.
- QSAR Toolbox, which was developed by the Laboratory of Mathematical Chemistry with the scientific and financial assistance of OECD and the European Union. It groups chemicals into categories and fills gaps in toxicity data in order to assess chemical hazards.

VEGA, Lazar, and QSAR Toolbox use carcinogenicity data from one or more of the following databases: the Distributed Structure-Searchable Toxicity (DSSTox) Public Database Network (US EPA, 2013), the Carcinogen Potency Database (Gold *et al.*, 2011) and ISSCAN (Istituto di Sanità database on chemical carcinogens) (Benigni *et al.*, 2008). The three models use mutagenicity data from one or more of the following databases: Benigni-Bossa mutagenicity rulebase, DSSTox, ISSCAN, bacterial mutagenicity ISSSTY, ECHA CHEM, genotoxicity OASIS, and Kazius-Bursi Salmonella

mutagenicity (Kazius *et al.*, 2005). Further details on the models and the databases are provided in Appendix D.

QSAR modeling results

Summary results from these models are discussed below and are provided in Table 30.

Table 30. Summary of QSAR model results for carcinogenicity and mutagenicity[1]

Endpoint	QSAR model		2,3-DAT	2,5-DAT	2,6-DAT	3,4-DAT	3,5-DAT
Carcinogenicity	VEGA CAESAR		+	+	+	+	-
	Lazar[2]		+	+	+	+[3]	+
	OECD Toolbox (mouse)[4]		-	NA	NA	-	NA
	OECD Toolbox (rat)[4]		NA	+	+	NA	+
Mutagenicity	VEGA	CAESAR	+	+	+	+	+
		SarPy	+	+	+	+	+
		ToxTree	+	+	+	+	+
	Lazar[5]		+	+	+	+	+[6]
	OECD QSAR Toolbox	Gene mutation *predictions*[4]	-	±[7]	-	-	±[8]
		Gene mutation *experimental data*	+	+	+	+	ND
		DNA damage/ repair[4]	-	NA	-[9]	NA	NA
		Chromosomal aberration[4]	-	+	±[10]	+[9]	+

NA: not available (prediction was out of the applicability domain); ND: no experimental data in the database

[1] Only predictions that were made with good reliability were included, as assessed by OEHHA

[2] Lazar provides a confidence value that indicates if a chemical falls within the applicability domain. Only predictions with confidence values > 0.05 are included. The reported prediction combines predictions made from two databases: 'DSSTox Carcinogenic Potency DBS SingleCellCall' and 'DSSTox ISSCAN v3a Canc'

[3] Prediction for 3,4-DAT includes two additional databases: 'DSSTox Carcinogenic Potency DBS MultiCellCall' and 'DSSTox Carcinogenic Potency DBS Mouse'

[4] OECD Toolbox indicates the strength of the prediction and a p-value for the prediction. Only moderate, strong, or very strong predictions with $p < 0.05$ are included

[5] Lazar provides a confidence value that indicates if a chemical falls within the applicability domain. Only predictions with confidence values > 0.05 are included. Prediction is based on 'DSSTox Carcinogenic Potency DBS Mutagenicity' database

[6] Prediction is based on 'DSSTox Carcinogenic Potency DBS Mutagenicity' and 'Kazius-Bursi Salmonella mutagenicity' database

[7] Seven predictions were negative and one prediction was positive.

[8] Seven predictions were negative and two predictions were positive

[9] Not a prediction, this is based on experimental data

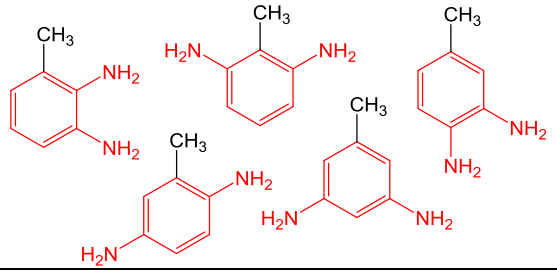
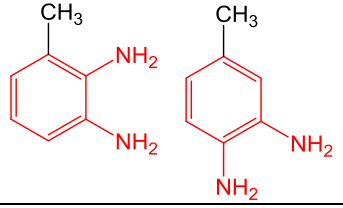
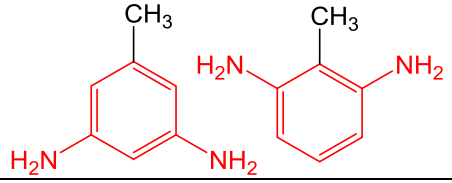
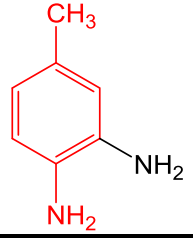
[10] Chromosomal aberration was predicted to be positive. The database also contained negative experimental chromosomal aberration results.

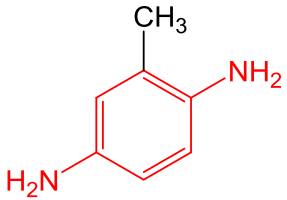
VEGA

CAESAR predicted 2,3-, 2,5-, 2,6- and 3,4-DAT to be carcinogenic, and 3,5-DAT to be non-carcinogenic with good reliability. ToxTree did not make any carcinogenicity predictions with good reliability because all isomers were outside of the applicability domain.

All the mutagenicity models (CAESAR, SarPy, and TOXTREE) predicted all DAT isomers to be mutagens. SarPy and ToxTree also identified structural alerts (SAs) associated with each DAT (Table 31). SarPy identified SA 70 for 2,3-DAT and 3,4-DAT; SA 105 for 2,5-DAT; SA 79 for 2,6-DAT; and SA 95 for 3,5-DAT. ToxTree identified SA 28 for all five DAT isomers.

Table 31. Structural alerts identified for the DAT isomers

DAT Isomer	SA	Name/ description of SA	Highlighted SA within each isomer	Source of SA
2,3-, 2,5-, 2,6-, 3,4-, and 3,5-DAT	28	Primary aromatic amine, hydroxyl amine and its derived esters		Benigni-Bossa structural alerts (Benigni *et al.*, 2008)
2,3-DAT and 3,4-DAT	70	Phenylenediamine		SarPy[1]
2,6-DAT	79	2,6-DAT		SarPy[1]
2,6-DAT and 3,5-DAT	95	*m*-Phenylenediamine		SarPy[1]
3,4-DAT	104	**p**-Toluidine		SarPy[1]

DAT Isomer	SA	Name/ description of SA	Highlighted SA within each isomer	Source of SA
2,5-DAT	105	*p*-Phenylenediamine		SarPy[1]

[1] SarPy indicates the structural alert as a SMILES string. SMILES strings were converted to structures using ChemSpider (http://www.chemspider.com/)

Lazar

Lazar predicted all five DAT to be carcinogenic. Predictions were made using two databases: 'DSSTox Carcinogenic Potency DBS SingleCellCall' and 'DSSTox ISSCAN v3a Canc'. 3,4-DAT used those two databases, in addition to 'DSSTox Carcinogenic Potency DBS MultiCellCall' and 'DSSTox Carcinogenic Potency DBS Mouse'. 'DSSTox Carcinogenic Potency DBS Mouse' contains carcinogenicity data for mice; the other three databases combine carcinogenicity data for mice and rats. All DAT isomers were predicted to be non-carcinogens in hamsters, but these predictions were unreliable and were not reported because the dataset of carcinogens tested in hamsters is very small.

Lazar predicted all five DAT isomers to be mutagenic based on the 'DSSTox Carcinogenic Potency DBS Mutagenicity' database. The 'Kazius-Bursi Salmonella mutagenicity' database also predicted 3,5-DAT to be mutagenic. Positive mutagenicity data was included in the 'Kazius-Bursi Salmonella mutagenicity' database for the other four DAT isomers.

OECD QSAR Toolbox

Toolbox predicted 2,3-DAT and 3,4-DAT to be non-carcinogenic in mice. The predictions for 2,5-DAT, 2,6-DAT, and 3,5-DAT were not reliable for mice. Toolbox predicted 2,5-DAT, 2,6-DAT, and 3,5-DAT to be carcinogenic in rats. The predictions for 2,3-DAT and 3,4-DAT were not reliable for rats. Predictions were unreliable if there were too few similar compounds with carcinogenicity data within the dataset or if the similar compounds had conflicting results, which then made the prediction weak.

Toolbox provided predictions for several mutagenicity endpoints: gene mutation, DNA damage/repair, and CA. As shown in Table 7 in Section 3.3.2, there were experimental data for some endpoints in the OECD QSAR Toolbox dataset, including *Salmonella* gene mutation results for 2,3-DAT, 2,5-DAT, 2,6-DAT, and 3,4-DAT, DNA damage and/or repair results for 2,6-DAT, and CA results for 2,6-DAT and 3,4-DAT. Some of the predictions contrasted with the experimental data.

Summary of Structure Activity Comparisons

All of the DAT isomers but 3,5-DAT have been tested in genotoxicity and *in vitro* cell transformation assays and found to be active, as have each of the four comparison monocyclic aromatic amines (with the exception of *p*-cresidine, which has not been

tested for *in vitro* cell transformation). In comparing the genotoxic activity observed for 2,4-DAT with that of the other DAT isomers, 2,3-, 2,5-, 2,6-, and 3,4-DAT[15] were also each mutagenic, 2,5- and 2,6-DAT also induced chromosomal effects, and 2,5-, 2,6- and 3,4-DAT also induced DNA damage or other DNA effects. Despite the limitations in the conduct and design of the animal cancer bioassays on 2,5-DAT and 2,6-DAT, increases in the incidence of tumors were observed for these two DAT isomers at sites in common with 2,4-DAT target tumor sites (2,5-DAT: lung tumors in mice, 2,6-DAT: liver tumors in rats and mice). With regard to QSAR model predictions of the carcinogenicity and mutagenicity of the DATs, while not all models run were in agreement, both the VEGA (with the exception of the carcinogenicity prediction for 3,5-DAT) and Lazar models predicted 2,3-, 2,5-, 2,6-, 3,4- and 3,5-DAT to be mutagenic and carcinogenic.

4. MECHANISMS

4.1 Genotoxicity

A body of evidence suggests that DATs may act via a genotoxic mechanism or mechanisms, as discussed in Section 3.3.2 (Genotoxicity) and summarized in Section 3.3.8 (Structure Activity Comparisons) of this document. The evidence includes positive findings for mutagenicity for each of the five isomers tested in *Salmonella* (2,3-DAT, 2,4-DAT, 2,5-DAT, 2,6-DAT, and 3,4-DAT[16]), and positive findings for four of the isomers (2,4-, 2,5-, 2,6-DAT, and 3,4-DAT) for either chromosomal effects or damage, binding, or changes in synthesis of DNA.

DATs, like most aromatic amines, appear to require metabolic activation for genotoxic and carcinogenic activity. Benigni and Passerini (2002) found similar QSARs for *Salmonella* mutagenicity and rodent carcinogenicity for aromatic amines as a class. Activation of the DATs is thought to occur via mixed function oxidase-catalyzed N-hydroxylation of an amino group. For example, Cunningham and Matthews (1990) found that following the N-hydroxylation of the amino group at the 4-position, the hydroxylamino intermediate of 2,4-DAT can then be activated by *o*-acetylation to proximate mutagens such as 4-acetoxyamino-2-aminotoluene. In another study by Cheung *et al.* (1996), N-hydroxylation of the diaminotoluenes (2,3-, 2,4-, 2,5-, and 2,6-DAT) by CYP1A was detected in rats. Among the four isomers, 2,5-DAT showed the

[15] 3,4-DAT was weakly positive in one study in *Salmonella*
[16] 3,4-DAT was weakly positive in one study in *Salmonella*

highest level of N-hydroxylation, followed by 2,4-DAT; 2,3- and 2,6- showed much lower N-hydroxylation. Ring hydroxylation may also occur (Waring and Pheasant, 1976). Hydroxylation may lead to the formation of DNA reactive metabolites, such as the highly reactive nitrenium ion, the quinone imine, and reactive oxygen species (Chao *et al.*, 2012) (Figure 3). Furthermore, both 2,4-DAT and 2,6-DAT also form free radicals in yeast, with the 2,6-isomer being more potent than the 2,4-isomer (Brennan and Schiestl, 1997).

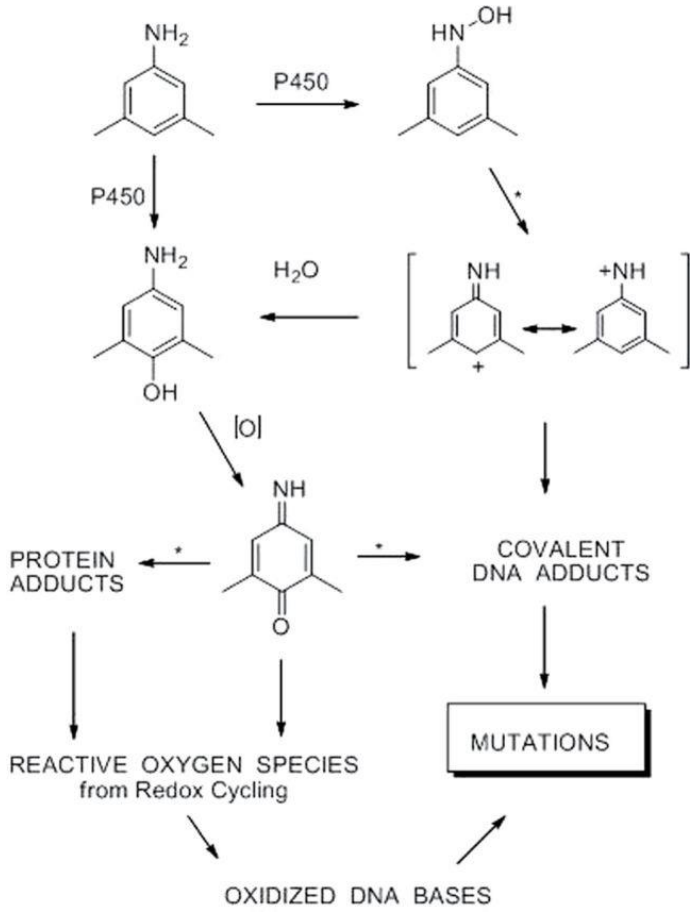


Figure 3. Three possible pathways of metabolic activation of dimethylanilines, adapted from (Chao *et al.*, 2012).

Mutagenic metabolites have been identified for two of the DAT isomers, as discussed in Section 3.3.1, Pharmacokinetics and Metabolism. 2,4-DAT is metabolized to 4-acetylamino-2-aminotoluene, which has been shown to be mutagenic in *Salmonella* (Sayama *et al.*, 2002). 2,6-DAT is metabolized to 5-hydroxy-2-acetylamino-6-aminotoluene and 2,6-diacetylaminotoluene, both of which are mutagenic in *Salmonella* (Cunningham *et al.*, 1989).

4.2 Other Possible Mechanisms

Other possible mechanisms by which DATs may be involved in carcinogenicity include:

- ***Receptor-mediated mechanisms***

AhR (Aryl hydrocarbon receptor)

There is evidence that some of the DAT isomers interact with the AhR. AhR is a cytosolic transcription factor that mediates the toxicity and carcinogenicity of numerous chemicals, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polycyclic aromatic hydrocarbons. Upon ligand binding and activation, AhR regulates the transcriptional activation of CYP1A1, CYP1A2, and CYP1B1, enzymes that are involved in the bioactivation of many carcinogens (Murray *et al.*, 2014; Go *et al.*, 2015). In addition to these cytochrome P450 genes, a number of other genes are regulated by AhR, including JUN, FOS, GSTM, UTG1A, ALDH1, and TNF-α (Puga *et al.*, 2005; Puga *et al.*, 2009). AhR plays an important role in cell proliferation, differentiation, hepatic and immune system homeostasis, and tumor development (Guo *et al.*, 2003).

In a competitive binding assay, 2,3-DAT and, to a lesser extent, 2,4-DAT displaced ^{3}H-radiolabeled TCDD from rat hepatic AhR and induced the expression of CYP1A enzymes, while no evidence of binding was observed for 2,5-DAT or 2,6-DAT (Cheung *et al.*, 1996). These findings are consistent with data from ToxCast, which indicate that 2,3-DAT, 2,4-DAT and 3,4-DAT are active in the upregulation of the transcriptional activity of AhR in HepG2 cells (see Section 3.3.7). In addition, 2,3-DAT was active in an enzyme reporter assay in ToxCast that indicates upregulation of CYP1A2 activity (see Appendix Table C1). Since CYP1A catalyzes the N-hydroxylation of DATs, AhR activation by these DAT isomers likely increases their bioactivation to reactive intermediates.

ToxCast data also provide information on the effect of some of the DAT isomers on the expression of other AhR target genes. For example, 2,3-DAT and 3,4-DAT upregulate the transcriptional activity at the activating protein-1 (AP-1) binding site, which is downstream of the AhR-regulated gene c-Jun (See Appendix Table C4).

These data suggest that 2,3-DAT, 2,4-DAT, and 3,4-DAT interact with the AhR and raise the possibility that DATs might act through AhR-mediated mechanisms.

Androgen Receptor (AR)

AR is a ligand activated receptor that translocates into the nucleus upon activation, and transcriptionally activates the expression of many target genes. Activation of AR has been linked to various cancers of the prostate, breast, bladder, liver, and pancreas in humans (Li *et al.*, 2012; Kanda *et al.*, 2014; Proverbs-Singh *et al.*, 2015). AR overexpression is a key mechanism of the development of castration-resistant prostate cancer (Waltering *et al.*, 2009). In a cell-free AR binding assay from ToxCast, 2,3-DAT was found to bind to the chimpanzee AR (See Appendix Table C1).

Estrogen Receptor (ER)

ER is a ligand activated nuclear receptor that is involved in human carcinogenesis (Shanle and Xu, 2010; Berger *et al.*, 2013). Activation of ER alpha (ERα) can lead to genomic activation of target gene transcription, as well as non-genomic activation of intracellular proliferation-promoting signaling pathways (e.g., PI3K/Akt, MAPK/ERK, and PKCδ) (Su *et al.*, 2014). Several ToxCast HTS assays examined the effect of 2,3-, 2,4-, and 3,4-DATs on ER activity. 2,3-DAT was found to be an agonist for the transcriptional activity of human ERα. 3,4-DAT was a strong agonist for ERα in an human ovarian cancer cell line (BG1 cells), and a weak ERα antagonist in a human embryonic kidney cell line (HEK293 cells) (See Appendix Table C3).

Peroxisome proliferator-activated receptors (PPARs)

PPARs are a group of nuclear receptors that function as transcription factors regulating gene expression, and are involved in the proliferation and progression of many cancers (Youssef and Badr, 2011). ToxCast data indicate that 2,3-DAT and 3,4-DAT upregulate the transcriptional activity at the PPAR response element (PPRE) in HepG2 cells, indicating activation of one or more of the PPARs (α, δ, γ) (see Section 3.3.7). In a ToxCast binding assay, 2,3-DAT was found to bind to the human PPARγ with high potency (AC_{50} < 0.001 µM) (See Appendix Table C1).

- ***Cell proliferation and cell cycle arrest***

Resisting cell death (such as apoptosis) and sustaining proliferative signaling are two of the six hallmarks of cancer (Hanahan and Weinberg, 2011). The evidence for DATs and cell proliferation is mixed, with some findings suggesting increased proliferation, and others suggesting decreased proliferation. Evidence from studies of cell proliferation and growth and molecular gene/protein expression indicate that 2,4- and 2,6-DATs disturb the normal cell cycle and proliferation.

After nine days of daily oral doses, 2,4-DAT but not 2,6-DAT induces hepatocellular cell proliferation in rats (Cunningham *et al.*, 1991). In an *in vitro* assay for cell proliferation, 2,6-DAT at 100 µM increased the proliferation of human lung fibroblasts that were seeded at low density, while shorter treatment with lower doses of 2,6-DAT significantly inhibited the viability of cells seeded in higher density (Pons *et al.*, 1999) (Section 3.3.5). The study authors proposed that the dual cytotoxic and growth-stimulatory effect by 2,6-DAT was due to the compensatory repair by the cells that survived the injury. ToxCast data suggest that the DATs may inhibit cell proliferation. Specifically, 2,3-, 2,4- and 3,4-DAT (at 10-20 µM) were each found to inhibit the proliferation of human skin fibroblast cells after 24 hours (Table 27), and 2,3- and 3,4-DAT (at 4-6 µM) were found to inhibit the growth of human T47D cells after 80 hours (See Appendix C4). The effect of 2,6-DAT on cell proliferation could be dependent on the type and density of cells, concentration of the chemical, and treatment time.

As discussed in Section 3.3.6, both 2,4-DAT and 2,6-DAT induced the expression of genes coding for cell-cycle related proteins such as Cyclin G1 and Cyclin-dependent kinase inhibitor 1A (p21) in rat liver. Cyclin G1 is a major regulator of G2/M arrest in response to DNA damage (Kimura *et al.*, 2001). The protein p21 is tightly regulated by the tumor suppressor p53 (Rodriguez and Meuth, 2006). It is known that activation of both p53 and p21 is required in the G2/M arrest in response to DNA damage (Bunz *et al.*, 1998). 2,6-DAT has been shown to cause DNA damage in various cells and animals (see Section 3.3.2), and marked cell cycle arrest in CHO cells (Gulati *et al.*, 1989), which is consistent with the data by Nakayama *et al.* (2006) (see Section 3.3.6) that two major regulators of the G2/M arrest are upregulated by 2,6-DAT. The G2/M checkpoint arrest can either enhance or reduce the cytotoxicity of xenobiotics (DiPaola, 2002), thus more data is needed to fully interpret the effect of 2,4-DAT and 2,6-DAT on the cell cycle.

The proposed mechanisms for the carcinogenicity of DATs have been summarized here in Table 32. Overall, the strongest mechanism for possible carcinogenicity of DATs comes from genotoxicity. Various *in vitro* mechanistic data indicate a range of activities of these isomers, from activation of receptor signaling to cell proliferation and cell cycle arrest.

Table 32. Overview of mechanistic data for the DAT isomers

Endpoint		Isomer					
		2,3-DAT	2,4-DAT	2,5-DAT	2,6-DAT	3,4-DAT	3,5-DAT
Genotoxicity	Mutagenicity	+	+	+	+	+/-[1]	NT
	Chromosomal effects	-	+	+	+	NT	
	DNA damage and other effects	NT	+	+	+	+[2]	
Receptor-mediated	AhR activation	+	+	-	-	+[3]	
	AR binding	+	NT	NT	NT	NT	
	ER activation[3]	+[3]	-[3]	NT	NT	+[3]	
	PPAR activation	+[3]	-[3]	NT	NT	+[3]	
Altered cell proliferation and cycle control	Induction of cell proliferation	-[3]	+[4]	NT	+[4]	-[3]	
	Cell cycle arrest	NT	+[5]	NT	+	NT	

NT, not tested

[1] Weakly positive in one study in *Salmonella*

[2] Positive in inhibiting testicular DNA synthesis by Greene *et al.* (1981), but the exact mechanism was not clearly shown by the study authors.

[3] Based on ToxCast HTS assays

[4] Cytotoxicity of these isomers was also observed. Effect on cell proliferation may depend on cell type and experimental protocol.

[5] Only based on the induction of the expression of cell cycle arrest related genes

5. REVIEWS BY OTHER AGENCIES

"Diaminotoluene (mixed)" has been reviewed and classified by US EPA as to its potential carcinogenicity as a Group B2 (probable human carcinogen) (US EPA, 1988). As discussed in section 2, the 1988 US EPA review and classification is the basis for the January 1, 1990 listing of "diaminotoluene (mixed)" as causing cancer under Proposition 65. 2,4-DAT was added to the Proposition 65 list as causing cancer via the State's Qualified Experts mechanism on January 1, 1988.

NIOSH (1989) reviewed and classified TDA (toluene diamine; diaminotoluene) and 2,4-TDA (2,4-DAT) as to their potential carcinogenicity, stating:

"The National Institute for Occupational Safety and Health (NIOSH) concludes that the data on carcinogenicity provide sufficient evidence to warrant concern about the potential consequences of occupational exposure to TDI [toluene diisocyanate] and TDA [toluene diamine]. The tumorigenic responses observed in both rats and mice treated with either TDI or TDA meet the criteria of the Occupational Safety and Health

Administration (OSHA) Cancer Policy for classifying a substance as a potential occupational carcinogen [29 CFR 1990]. Although the carcinogenic potential of the other TDI and TDA isomers has not been adequately determined, exposure to all TDI and TDA isomers should be reduced. NIOSH therefore recommends that all the isomers of TDI and TDA be regarded as potential occupational carcinogens and that occupational exposures to TDI and TDA be limited to the lowest feasible concentrations. The potential for TDI- or TDA-induced cancer in humans has not been determined, but the risk of developing cancer should be decreased by reducing exposure to TDI and TDA in the workplace." (NIOSH, 1989, p. v)

"NIOSH therefore concludes that commercial grade TDI and 2,4-TDA are potential occupational carcinogens." (NIOSH, 1989, p. 7)

"Exposure to TDI and TDA has been shown to produce benign and malignant tumors in rats and mice. NIOSH therefore recommends that all TDI and TDA isomers or mixtures of isomers be regarded as potential occupational carcinogens in conformance with the OSHA Cancer Policy [29 CFR 1990]. Though evidence does not exist to demonstrate the carcinogenicity of all TDI and TDA isomers, the NIOSH recommendation applies to all of them and to mixtures of these isomers because of the gravity of the potential health effect (cancer) and because TDI and TDA rarely, if ever, occur as pure isomers in the workplace." (NIOSH, 1989, p. 8)

NTP reviewed and classified 2,4-DAT as reasonably anticipated to be a human carcinogen in the Second Annual Report on Carcinogens in 1981 (NTP, 2014a). NTP has not reviewed or classified the DATs as a group, or any other DAT isomers as to their potential carcinogenicity.

IARC (1978) initially reviewed the carcinogenicity of 2,4-DAT and 2,5-DAT in Volume 16 of the IARC Monographs series. In 1987, IARC classified 2,4-DAT as a Group 2B carcinogen (possibly carcinogenic to humans) and 2,5-DAT in Group 3 (not classifiable as to its carcinogenicity to humans) (IARC, 1987). IARC has not reviewed or classified the DATs as a group, or any other DAT isomers as to their potential carcinogenicity.

6. SUMMARY AND CONCLUSIONS

6.1 Summary of Evidence

No studies in humans were identified in the literature specifically designed to investigate the risk of cancer associated with exposure to one or more of the DAT isomers. Several cancer epidemiology studies have been conducted in populations exposed to complex mixtures that contain one or more DAT isomers and that also contain other compounds with known or suspected carcinogenic activity. These populations include individuals exposed to 2,4-DAT and/or 2,5-DAT (or its sulfate salt) in hair dye formulations through either occupational or personal use, individuals exposed to mixtures of 2,4-DAT and 2,6-DAT during polyurethane foam production and handling, and individuals with polyurethane-coated breast implants, which may degrade and release 2,4-DAT and 2,6-DAT. Because the exposures in these studies were to mixtures of chemicals (including other carcinogens), and because these studies often lack specific information on 2,4-DAT and/or 2,5-DAT exposure levels, they are very limited in their ability to assess the relationship between exposures to DATs and cancer risk.

Long-term carcinogenicity studies of 2,4-DAT, 2,5-DAT sulfate, and 2,6-DAT dihydrochloride have been conducted in rats and mice exposed via feed. No carcinogenicity studies were identified on 2,3-, 3,4-, or 3,5-DAT. Tumor findings from animal carcinogenesis bioassays on the three DAT isomers tested are as follows.

2,4-DAT:
Tumors were observed at a number of sites and in two species from four *s.c.* injection studies in rats, four dietary studies in rats, and four dietary studies in mice. The tumor findings are:

- Alveolar bronchiolar carcinomas in male B6C3F1 mice (NCI, 1979)
- Bone osteosarcoma in female Fischer rats (Cardy, 1979)
- Liver tumors in male and female Fischer rats (Cardy, 1979; NCI, 1979), male CD rats (Weisburger *et al.*, 1978), male Wistar rats (Umeda, 1955), female CD-1 mice (Weisburger *et al.*, 1978), and female B6C3F1 mice (NCI, 1979)
- Lymphomas in female B6C3F1 mice (NCI, 1979)
- Mammary gland tumors in male and female Fischer rats (Cardy, 1979; NCI, 1979)
- Subcutaneous fibromas in male and female Fischer rats (NCI, 1979) and male CD rats (Weisburger *et al.*, 1978)
- Subcutaneous sarcomas in male and female rats (strain not specified) (Umeda, 1955)
- Vascular tumors in male and female CD-1 mice (Weisburger *et al.*, 1978)

2,5-DAT:

- In the male F344 rat study by NCI (1978), the incidence of testicular interstitial cell tumors was significantly increased in the 600 ppm dose group compared to its control group ($p<0.05$), and in the 2000 ppm dose group compared to its control group ($p<0.05$).
- In the female B6C3F1 mouse study by NCI (1978), the incidences of lung alveolar/bronchiolar adenoma and combined adenoma and carcinoma were significantly increased ($p<0.05$) in the 1000 ppm dose group compared to its control group.

In its peer review of the NCI bioassays on 2,5-DAT sulfate, the Clearinghouse on Environmental Carcinogens (a NCI advisory body) noted, “the compound warranted further testing because of the experimental design and study conduct deficiencies, as well as the fact that 2,5-Toluenediamine [2,5-DAT] Sulfate had been shown to be positive in the Ames assay”, and suggested the compound be considered for retesting (CEC, 1978).

2,6-DAT:

- In the male F344 rat study by NCI (1980), significant dose-response trends were observed in the incidences of hepatocellular adenoma ($p<0.05$) and combined adenoma and carcinoma ($p<0.05$).
- In the male F344 rat study by NCI (1980), a significant dose-response trend was observed in the incidence of pancreatic islet-cell adenoma ($p<0.05$).
- In the female B6C3F1 mouse study by NCI (1980), a significant dose-response trend was observed in the incidence of hepatocellular carcinoma ($p<0.05$), but not combined adenoma and carcinoma.

In its peer review of the NCI bioassays on 2,6-DAT dihydrochloride, a member of the Clearinghouse on Environmental Carcinogens noted that a dose-related incidence of liver tumors occurred in treated male rats and female mice, but opined that the maximum tolerated dose was not reached (CEC, 1980). He noted that mice receiving the 2,4-DAT isomer in other long-term studies conducted by NCI, were given about twice the dose used in these studies. In light of these observations, he recommended the report not be accepted. To address concerns raised by the study deficiencies the Clearinghouse recommended that a statement be added to the report indicating the need for further testing of this compound.

A body of evidence suggests that DATs may act via a genotoxic mechanism or mechanisms. The evidence includes positive findings for mutagenicity for each of the five isomers tested in *Salmonella* (2,3-, 2,4-, 2,5-, 2,6-, and 3,4-DAT[17]), and positive findings for four of the isomers (2,4-, 2,5-, 2,6-, and 3,4-DAT) for either chromosomal effects or damage, binding, or changes in synthesis of DNA. No genotoxicity data are available for 3,5-DAT. The specific findings for the five tested DAT isomers are as follows:

2,3-DAT

Positive findings were observed for 2,3-DAT in each of the three studies conducted in *Salmonella typhimurium* but not in the one *in vivo* study conducted in mice. Specifically, 2,3-DAT:

- Induced mutations in the *Salmonella* reverse mutation assay with metabolic activation by induced and non-induced rat liver microsomes

2,4-DAT

Positive findings were observed in multiple bacterial mutagenicity tests (Table 11) and in multiple *in vitro* mammalian genotoxicity assays and in studies assessing DNA adduct formation in cell free systems (Table 12). *In vivo*, 2,4-DAT induced mutations, DNA damage, and DNA adducts, UDS, DNA fragmentation, and MN. 2,4-DAT did not induce dominant lethal mutations in mice (Table 13). Briefly, 2,4-DAT:

- Induced mutations in L5178Y mouse lymphoma cells (in the absence of rat liver S-9) and AT3-2 CHO cells (in the presence or absence of S-9) at the autosomal *tk* locus, but not at the sex-linked *hgprt* locus in either cell line
- Damaged DNA (induced DNA-strand breaks) in human HepG2 cells and a 3-D reconstructed human epidermal cell model
- Formed adducts with calf thymus single-stranded DNA in a cell-free system (in the presence of S-9) and covalently bound to DNA in rat primary hepatocytes
- Induced MN in human HepG2 cells
- Induced CAs and SCEs in CHO cells in the presence and absence of rat liver S-9
- Induced UDS in human HepG2 cells, primary human hepatocytes, primary rat hepatocytes, and cultured rat thoracic aortic smooth muscle cells
- Induced mutations in *Drosophila melanogaster*, causing dominant mutations and sex-linked recessive mutations in F_1 and F_2 offspring, respectively, and in the liver and kidneys in mice and the liver in rats *in vivo*.

[17] 3,4-DAT was weakly positive in one study in *Salmonella*

- Damaged DNA (induced DNA-strand breaks) in rats and mice in multiple tissues (brain, stomach, colon, kidneys, and liver in rats, and stomach lung, liver, and kidneys in mice) *in vivo*.
- Formed DNA adducts in rats in the liver, mammary gland, mammary fat pads, kidneys, and lungs, but not in T-lymphocytes from the spleen *in vivo*.
- Induced UDS in rat liver in one of two studies *in vivo*
- Induced MN in rat liver in five studies and in rat bone marrow cells in one of four studies *in vivo*
- Induced SCEs in mouse bone marrow cells *in vivo*
- Inhibited DNA synthesis in mouse testes *in vivo*

2,5-DAT

Positive findings were observed in a number of bacterial assays (Table 14), *in vitro* mammalian cell assays (Table 15), and *in vivo* assays in rats and mice with 2,5-DAT (or its sulfate or dihydrochloride salt) (Table 16). In summary, 2,5-DAT:

- Induced mutations (in the presence of S9 but not without S9) in the *Salmonella* reverse mutation assay in test strains TA98, 100, 102, 1535, 1537, and 1538 (Table 14) and the mutagenicity increased significantly (about 40 fold) with hydrogen peroxide pretreatment
- Induced DNA damage in *Salmonella* in the umu test with S-9 metabolic activation
- Induced UDS in rat and hamster hepatocytes *in vitro*
- Induced DNA damage (Comet assay) in hamster and human cells *in vitro*
- Induced CA in two different hamster cell lines
- Induced DNA damage (Comet assay) in the stomach in rats *in vivo*
- Inhibited DNA synthesis in mouse testes *in vivo*

2,6-DAT

Positive findings were observed in a number of bacterial assays, one study in yeast (Table 17), multiple *in vitro* mammalian cell assays (Table 18), and several *in vivo* assays in rats and mice with 2,6-DAT (or its dihydrochloride salt) (Table 19). Specifically, 2,6-DAT:

- Was positive in the *Salmonella* reverse mutation assay (in the presence of S-9 but not without S-9) with test strains TA98, TA100, TA1538, YG1024, and YG1029
- Induced DNA damage in *Salmonella* in the umu test using the test strain NM2009 and in the presence of S-9
- Induced intrachromosomal recombination in *Saccharomyces cerevisiae* (without S-9, but not in the presence of S-9)

- Was mutagenic in L5178Y mouse lymphoma cells at the *tk* locus in the absence of S-9
- Bound covalently to primary rat hepatocyte DNA
- Induced UDS in primary human hepatocytes and in cultured rat thoracic aortic smooth muscle cells
- Induced CA in CHO cells (in the absence of S-9) and in Chinese hamster lung cells (with or without S-9)
- Induced SCEs and MN in CHO cells (in the presence or absence of S-9)
- Formed DNA adduct in rat liver[18] *in vivo*
- Caused DNA damage (Comet assay) in rat liver *in vivo*
- Induced UDS in rat liver *in vivo*
- Induced MN in in rat bone marrow *in vivo*
- Inhibited DNA synthesis in mouse testes *in vivo*

3,4-DAT
Positive findings were observed in one of three studies in *Salmonella typhimurium* and in one *in vivo* study conducted on 3,4-DAT. Specifically, 3,4-DAT:

- Was weakly positive in the TA1538 reverse mutation assay in the presence of rat liver microsomes induced with β-NF (Table 20)
- Inhibited DNA synthesis in mouse testes *in vivo* (Table 21)

The pharmacokinetics and metabolism of 2,4-, 2,5-, and 2,6-DAT have been studied on humans, monkeys, mice, rats, dogs, hamsters, guinea-pigs, and rabbits via various *in vivo* routes of administration: dermal (primary), oral gavage, *i.p.* injection, *i.v.* infusion, and *s.c.* injection. Additionally, *in vitro* absorption measurements of 2,5-DAT using human and pig skin and *in vitro* metabolism studies on 2,4- and 2,5-DAT were available. DATs are rapidly and extensively absorbed following oral ingestion. Dermal absorption efficiency and rate are highly dependent on experimental design and animal species tested. Upon intake, DATs are rapidly and widely distributed throughout the body without signs of accumulation in any organs. DATs and their metabolites are eliminated mainly in the urine within a day of exposure, and are eliminated in the feces in smaller amounts. Three major metabolic pathways have been identified for the DATs: N-acetylation to mono- and di-acetylamino metabolites, methyl-oxidation to α-hydroxy metabolites and benzoic acid metabolites, and ring-hydroxylation to phenolic metabolites. These metabolic pathways also occur in combination to form various metabolites. Although glucuronidation of the hydroxy-metabolites may occur, no studies

[18] Weakly positive: positive adduct spot on the autoradiogram only with extended autoradiogram exposure time (14 h)

investigating this pathway have been reported. Mutagenic metabolites have been identified for 2,4- and 2,6-DAT, namely 4-acetylamino-2-aminotoluene (a 2,4-DAT metabolite) and 5-hydroxy-2-acetylamino-6-aminotoluene and 2,6-diacetylaminotoluene (2,6-DAT metabolites). All three are mutagenic in bacterial systems.

A number of *in vitro* cell transformation studies have been conducted with 2,4-DAT, 2,5-DAT, 2,6-DAT, and 3,4-DAT in SHE and Bhas 42 cells, and 2,3-DAT has been tested in one set of studies in Bhas 42 cells (Table 22 and Table 23).

- 2,4-DAT was positive in 11 out of 12 *in vitro* cell transformation studies conducted with SHE cells
- 2,4-DAT was positive in Bhas 42 cells in the 'initiation' cell transformation assay, but not the 'promotion' assay
- 2,5-DAT, 2,6-DAT, and 3,4-DAT were weakly positive in one set of *in vitro* cell transformation studies conducted with SHE cells (Table 23)
- 2,3-DAT, 2,5-DAT, and 3,4-DAT were positive in Bhas 42 cells in the 'promotion' cell transformation assay, but not the 'initiation' assay
- 2,6-DAT was negative in Bhas 42 cells in both the 'initiation' and 'promotion' cell transformation assays

In an *in vivo* assay, 2,4-DAT, but not 2,6-DAT (dihydrochloride salt), induced hepatocellular proliferation after nine days of oral dosing in rats (Table 24). In an *in vitro* assay 2,4-DAT was cytotoxic to human lung fibroblasts, while 2,6-DAT exhibited both cytotoxic and proliferative effects.

Changes in gene or protein expression have been associated with 2,3-, 2,4-, or 2,6-DAT treatment in one or more *in vivo* or *in vitro* assays.

- Microarray expression analysis examined the effect of *in vivo* exposure to 2,4- or 2,6-DAT for 28 days in rats. Data were presented for 54 genes, which are involved in many biological processes, including cell cycle control and apoptosis (Table 25 and Appendix B). The effect of 2,4-DAT exposure on rat liver gene expression was generally greater than that of 2,6-DAT.
- In an *in vitro* assay with immature dendritic cell-like cells, 2,5-DAT decreased the expression of Aquaporin 3 mRNA and increased expression of Interleukin-1B mRNA.
- In an *in vivo* assay, 2,3- and 2,4-DAT induced the expression of CYP1A protein in the hepatocytes of treated rats, while 2,5- and 2,6-DAT did not.
- In an *in vitro* assay in Balb/c 3T3 cells, 2,4-DAT induced the expression of CYP1A1.

2,3-, 2,4-, and 3,4-DAT have been tested in US EPA ToxCast HTS assays, and were active in 70/421, 7/392, and 49/405 assays, respectively (Section 3.3.7 and Appendix C). There are four assays in which all three DATs were active, and the endpoints of these assays include upregulation of AhR transcription activity (two assays), upregulation of thrombomodulin expression, and inhibition of the proliferation of human skin fibroblasts (Table 27). The effects on AhR activation are consistent with the data from a competitive binding study in which either 2,3-DAT or 2,4-DAT displaced ^{3}H-radiolabelled TCDD from rat hepatic AhR and induced the expression of CYP1A, a target of AhR.

Other observations from the ToxCast assays include the following:

- 2,3-DAT binds to the chimpanzee AR in a cell free assay, and may activate the AR
- 2,3-DAT is an ERα agonist, inducing human ERα transcriptional activity
- 3,4-DAT is a strong ERα agonist in a human ovarian cancer cell line, and a weak ERα antagonist in a human embryonic kidney cell line
- 2,3-DAT binds to human PPARγ, and 2,3- and 3,4-DAT activate one or more PPARs (α, δ, and γ)
- 2,3- and 2,4-DAT inhibit human protein tyrosine phosphatase receptor type C, which may result in the activation of oncoproteins
- 2,4-DAT and 3,4-DAT inhibit brain-type creatine kinase, which may affect cell energy homeostasis

Biological activities of the DAT isomers thought to be relevant to carcinogenicity were compared amongst themselves and with four structurally related chemicals. QSAR models were also applied to predict the carcinogenicity and mutagenicity of the DAT isomers. All of the DAT isomers but 3,5-DAT have been tested and found to be active in genotoxicity and *in vitro* cell transformation assays, as have each of the four comparison monocyclic aromatic amines (with the exception of *p*-cresidine, which has not been tested for *in vitro* cell transformation). In comparing the genotoxic activity observed for 2,4-DAT with that of the other DAT isomers, 2,3-, 2,5-, 2,6-, and 3,4-DAT[19] were also each mutagenic, 2,5- and 2,6-DAT also induced chromosomal effects, and 2,5-, 2,6- and 3,4-DAT also induced DNA damage or other DNA effects. Despite the limitations in the conduct and design of the animal cancer bioassays on 2,5-DAT and 2,6-DAT, increases in the incidence of tumors were observed for these two DAT isomers at sites in common with 2,4-DAT target tumor sites (2,5-DAT: lung tumors in mice, 2,6-DAT: liver tumors in rats and mice). With regard to QSAR model predictions

[19] 3,4-DAT was weakly positive in one study in *Salmonella*

of the carcinogenicity and mutagenicity of the DATs, while not all models run were in agreement, both the VEGA and Lazar models predicted 2,3-, 2,5-, 2,6-, and 3,4-DAT to be mutagenic and carcinogenic. The Lazar model predicted 3,5-DAT to be mutagenic and carcinogenic.

6.2 Conclusion

The evidence for the carcinogenicity of the DATs comes from cancer bioassays in animals (2,4-, 2,5-, and 2,6-DAT), genotoxicity assays (2,3-, 2,4-, 2,5-, 2,6-, and 3,4-DAT), *in vitro* cell transformation studies (2,3-, 2,4-, 2,5-, 2,6-, and 3,4-DAT), metabolism studies of 2,4-, 2,5- and 2,6-DAT, cell proliferation studies (2,4- and 2,6-DAT), gene expression studies (2,4-, 2,5-, and 2,6-DAT), protein expression studies (2,3-, 2,4-, 2,5-, and 2,6-DAT), ToxCast HTS assays (2,3-, 2,4-, and 3,4-DAT), structure-activity considerations, and QSAR predictions of carcinogenic and mutagenic activities.

- 2,3-DAT – observations following treatment include:
    - Mutations in *Salmonella*
    - Positive in Bhas 42 cells in the 'promotion' cell transformation assay
    - Induction of CYP1A protein expression
    - Findings from ToxCast HTS assays, such as effects on gene expression and transcription factor activities

- 2,5-DAT – observations following treatment include:
    - Testicular interstitial cell tumors in male rats (significant by pairwise comparison with controls at the low and high dose)
    - Lung alveolar/bronchiolar adenoma and carcinoma (combined) in female mice (significant by pairwise comparison with controls at the high dose)
    - Mutations and DNA damage in bacteria
    - UDS, DNA damage, and CA in mammalian cells
    - DNA damage and inhibition of DNA synthesis in animals
    - Positive in *in vitro* cell transformation assays in SHE cells and in Bhas 42 cells in the 'promotion' cell transformation assay

- 2,6-DAT – observations following treatment include:
    - Significant dose-response trends for hepatocellular adenoma and adenoma and carcinoma (combined) in male rats
    - Significant dose-response trend for pancreatic islet-cell adenoma in male rats

    - Significant dose-response trend for hepatocellular carcinoma, but not adenoma and carcinoma combined in female mice
    - Mutations and DNA damage in bacteria
    - Chromosomal recombination in yeast
    - Mutations, covalent binding to DNA, UDS, MN, SCEs, and CA in mammalian cells
    - DNA damage, DNA adducts, MN, UDS, and inhibition of DNA synthesis in animals
    - Two mutagenic metabolites, 5-hydroxy-2-acetylamino-6-aminotoluene and 2,6-diacetylaminotoluene, have been identified
    - Weakly positive in one *in vitro* SHE cell transformation assay

- 3,4-DAT – observations following treatment include:
    - Mutations in bacteria (weakly positive)
    - Inhibition of DNA synthesis in animals
    - Weakly positive in one *in vitro* cell transformation assay in SHE cells and positive in Bhas 42 cells in the 'promotion' cell transformation assay
    - Findings from ToxCast HTS assays, such as effects on gene expression and transcription factor activities

All the isomers bear close structural similarity to 2,4-DAT, which is already listed under Proposition 65 and not under reconsideration.

The findings for 2,4-DAT are:

- Tumor findings:
    - Alveolar bronchiolar carcinomas in male B6C3F1 mice
    - Bone osteosarcoma in female Fischer rats
    - Liver tumors in male and female Fischer rats, male CD rats, male Wistar rats, female CD-1 mice, and female B6C3F1 mice
    - Lymphomas in female B6C3F1 mice
    - Mammary gland tumors in male and female Fischer rats
    - Subcutaneous fibromas in male and female Fischer rats and male CD rats
    - Subcutaneous sarcomas in male and female rats (strain not specified)
    - Vascular tumors in male and female CD-1 mice
- One mutagenic metabolite, 4-acetylamino-2-aminotoluene, was identified in rats
- DNA adducts *in vivo* and genotoxic in bacteria, mammalian cells, and animals
- Induction of cell transformation *in vitro*, and cell proliferation in rat liver *in vivo*
- Altering the expression of cell cycle and apoptosis related genes, induction of CYP1A protein

- Findings from ToxCast HTS assays, including activation of receptors such as AhR, AR, ER, and PPARs

There are some structure-activity similarities among all six DAT isomers, and between the DATs and four comparison monocyclic primary aromatic amines (namely *p*-cresidine, 2,6-xylidine, *o*-toluidine, and *o*-phenylenediamine) that have been listed as carcinogens under Proposition 65. QSAR models were run on 2,3-, 2,5-, 2,6-, 3,4- and 3,5-DAT. Both the VEGA (with the exception of the carcinogenicity prediction for 3,5-DAT) and Lazar models predicted each of the five isomers to be mutagenic and carcinogenic.

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Appendix A. Epidemiological Studies on Cancer Risk from Exposure to Complex Mixtures Containing One or More DATs

A1 Epidemiological Studies on cancer risk from occupational or personal use of hair dyes

In the US, 2,4-DAT was used in hair dyes as a coupler until 1971, whereas 2,5-DAT and 2,5-DAT sulfate are common ingredients in permanent hair dyes (IARC, 2010) (see Table A1 below). 2,5-DAT and 2,5-DAT sulfate are used as "primary intermediates" to be oxidized on the hair by a "developer" such as hydrogen peroxide. The intended maximum on-head concentration is 2.0 percent for 2,5-DAT and 3.6 percent for 2,5-DAT sulfate (SCCS, 2012). Other primary intermediates used in permanent hair dyes include aminodiphenylamines, aminomethylphenols, and *para*-aminophenol (IARC, 2010).

Table A1. Ingredients of different types of hair dye, from Saitta *et al.* (2013)

Hair dye type	Ingredients
Temporary	**Azo derivatives** **Azine derivatives** **Thiazine derivatives** **Indoamines** **Triphenylmethane**
Semi-permanent	**Nitroanilines** **Nitrophenylenediamines** **Nitroaminophenols** **Azo derivatives** **Anthraquinone**
Permanent	***para*-Phenylenediamine** **2,5-DAT (*para*-toluenediamine)** **Substituted *para*-diamines** ***Ortho*- or *para*-aminophenols**

Hair dye ingredients that have been listed under Proposition 65 as causing cancer include:

- 4-Aminobiphenyl
- 4-Amino-2-nitrophenol
- 4-Chloro-*o*-phenylenediamine
- 2,4-DAT
- 4-Methoxy-*m*-phenylenediamine (2,4-diaminoanisole)

- *o*-Phenylenediamine (2-aminoaniline)

In 1978, IARC first reviewed the epidemiology studies on cancer risk and occupational exposure to or personal use of hair dye (IARC, 1978) and concluded:

- The epidemiological evidence suggests an elevated risk for both users of hair dyes and those with occupational exposure (barbers and hairdressers) to hair preparations.
- For users of hair dyes, the results are equivocal, since only one site (breast) has been studied.
- For persons with occupational exposure to hair-care products, including dyes, there is more evidence for an increased risk of cancer at certain sites.
- Further epidemiological studies, which should include workers employed in the production of hair dyes, are necessary before any firm conclusions can be drawn.

Since 1978, IARC has reviewed the carcinogenicity of hair dyes in 1993 and then again in 2010 (IARC, 1993; IARC, 2010). The 1993 Monograph (Volume 57) is superseded by the 2010 Monograph (Volume 99), which provides a comprehensive review regarding the cancer risk from occupational exposures of hairdressers and barbers and personal use of hair colourants (IARC, 2010). See the Attachment for the 2010 IARC Monograph.

Regarding the human evidence, IARC (2010) concluded:

- There is *limited evidence* in humans for the carcinogenicity of occupational exposures as a hairdresser or barber.
- There is *inadequate evidence* in humans for the carcinogenicity of personal use of hair colorants.

Cancer epidemiology studies published since the 2010 IARC review on occupational and personal use exposures to hair dyes are briefly summarized below. Findings related to bladder cancer are summarized first, followed by findings related to cancers at other sites.

Bladder Cancer

An additional four meta-analyses, three case-control studies, and one cohort study of bladder cancer were identified.

Meta-analyses

- Kelsh *et al.* (2008): This meta-analysis of 11 case-control studies and one cohort study found no association between personal hair dye use and bladder cancer in men, women, or both sexes combined. Among the studies that included data on permanent hair dye usage, no statistically significant association was found.
- Takkouche *et al.* (2009): This meta-analysis included 247 studies on the risk of various cancers among hairdressers. The 34 studies on bladder cancer gave a statistically significant pooled risk ratio (RR) = 1.3 (95% Confidence Interval (CI) 1.2-1.42). The RR was higher after adjustment for smoking.
- Harling *et al.* (2010): This meta-analysis on 42 studies in hairdressers found increased risk of bladder cancer, with summary risk ratios (SRRs) = 1.3 (95% CI 1.15-1.48) for "ever registered as hairdresser" and 1.70 (95% CI 1.01-2.88) for "job held ≥ 10 years". Adjustment for smoking did not significantly change the results.
- Turati *et al.* (2014): This meta-analysis included 15 case-control and 2 cohort studies, with over 8,504 bladder cancer cases/deaths. There was no increased risk of bladder cancer with personal use of any type of hair dye. Based on the seven studies that reported data on permanent hair dye, the pooled RR of bladder cancer for personal use of permanent hair dye was 0.92 (95% CI 0.77-1.09).

Case-control Studies

- Shakhssalim *et al.* (2010): This case-control study performed in Iran included 692 confirmed cases of bladder cancer and 692 individually matched controls. Hair dye use was significantly associated with bladder cancer, with an odds ratio (OR) = 1.81 (95% CI 1.08-3.06) (p=0.02).
- Koutros *et al.* (2011): A population based case-control study examined 1,193 cases of bladder cancer patients and 1,418 controls in the New England area between 2001 and 2004. While no association was found between hair dye use and bladder cancer risk overall, increased risks were observed for certain subgroups. In women who use permanent hair dyes (which typically contain 2,5-DAT) and had a college degree, the risk of bladder cancer was OR = 3.3 (95% CI 1.2-8.9). The risk was higher in women who exclusively use permanent hair dye and had a *NAT2* slow acetylation phenotype OR = 7.3 (95% CI 1.6-32.6).
- Ros *et al.* (2012): The association between personal use of permanent hair dye and the risk of bladder cancer was examined in a population-based case-control study in The Netherlands, with 1,385 cases and 4,754 controls. The OR for

bladder cancer risk with personal use of permanent hair dyes was 0.87 (95% CI 0.65-1.18).

Cohort Study

- Mendelsohn *et al.* (2009): In a prospective cohort of 70,366 Chinese women, the relative risk of bladder cancer with personal use of hair dyes was 1.14 (95% CI 0.56-2.35).

Cancers at other sites

An additional five meta-analyses, five case-control studies, and one cohort study were identified.

Meta-analyses

- Takkouche *et al.* (2009): This meta-analysis of cancer risks at various sites among hairdressers included 247 studies and is an update of Takkouche *et al.* (2005). The pooled RR of occupational exposure as a hairdresser was 1.27 (95% CI 1.15-1.41) for lung cancer, 1.52 (95% CI 1.11-2.08) for larynx cancer, and 1.62 (95% CI 1.22-2.14) for multiple myeloma.
- Shao *et al.* (2013): This is a meta-analysis on glioma risk and personal use of hair dyes. Five of the six studies included in this meta-analysis were reviewed by IARC (2010). The pooled RRs of glioma risk for users of any hair dyes were: RR = 1.132 (95% CI 0.887-1.446) for all six studies, RR = 1.291 (95% CI 0.938-1.777) for the four case-control studies and RR = 0.903 (95% CI 0.774-1.054) for the two cohort studies. No significant associations were observed with permanent hair dye use or duration of any hair dye use.
- Olsson *et al.* (2013): This is a meta-analysis of lung cancer risk among hairdressers with 19,369 lung cancer cases and 23,674 matched hospital or population controls pooled from 16 case-control studies over 1985-2010. The four studies that were not part of the IARC (2010) review have been summarized in this section. Based on the meta-analysis, the risk for lung cancer in women hairdressers was not significant after adjustment for smoking with OR = 1.12 (95% CI 0.75-1.68). Women hairdressers employed before 1954 had an increased risk of lung cancer with OR = 2.66 (95% CI 1.09-6.47) after adjustment for smoking. Male long time barber hairdressers (employed for more than 26 years) had a significantly increased risk of adenocarcinoma of the lung with OR = 2.20 (95% CI 1.02-4.77).
- Two pooled analyses on two subtypes of Non-Hodgkin's lymphoma (NHL)
    - Cerhan *et al.* (2014): This is a pooled analysis of 19 studies on diffuse large B-cell lymphoma (DLBCL) with 4,667 cases and 22,639 controls.

Occupational exposure in hairdressers was significantly associated with elevated risk of DLBCL: OR = 1.65 (95% CI 1.12-2.41). Personal use of hair dyes for over 20 years was associated with the mediastinal subtype of DLBCL: OR = 4.97 (95% CI 1.63-15.15).

- Linet *et al.* (2014): This is a pooled analysis of 19 studies on follicular lymphoma with 3,530 cases and 22,639 controls. Overall hair dye use was not associated with increased follicular lymphoma risk. There was a modestly elevated risk of follicular lymphoma in those who used hair dyes before 1980, OR = 1.40 (95% CI 1.10-1.78).

Case-control Studies

- Lung cancer case-control studies (each are included in the Olsson *et al.* (2013) meta-analysis)
  - Consonni *et al.* (2010): This population-based case-control study examined the association between lung cancer risk and various occupations including hairdressers in 2,100 lung cancer cases and 2,120 controls in Northern Italy. For both men and women hairdressers, the risks for lung cancer were elevated but not statistically significant: OR = 1.77 (95% CI 0.56-5.59) for men and OR = 2.22 (95% CI 0.64-7.79) for women.
  - Guida *et al.* (2011): This is a French population-based case-control study with 2,923 cases of lung cancer and 3,555 controls. There was an increased risk of lung cancer in women hairdressers with an OR = 2.0 (95% CI 0.7-5.7).
  - Tse *et al.* (2012): This is a population-based case-control study in Chinese men in Hong Kong from 2004 to 2006. There were 1,208 lung cancer cases and 1,069 age-matched controls. No elevated lung cancer risk was reported with occupational exposure for hairdressers.
- Lymphoma case-control studies
  - Wong *et al.* (2010): This case-control study included 649 NHL cases and 1298 individually matched controls from 25 hospitals in Shanghai, China. Use of hair dyes was not associated with increased risk for NHL with an OR = 0.93 (95% CI 0.75-1.16), or with any individual subtype of NHL.
  - Guo *et al.* (2014): This case-control study examined 24 single nucleotide polymorphisms (SNPs) in 16 DNA repair genes among 518 NHL cases and 597 controls and evaluated the associations between hair dye use and risk of overall NHL and common subtypes. The results showed that women who used hair dye before 1980 had significantly increased risk of NHL, especially follicular lymphoma, if they carried certain SNPs.

Cohort Studies

- Mendelsohn *et al.* (2009): This is a prospective cohort study on personal use of hair dyes and cancer risk in 70,366 Chinese women with an average of 7 years follow-up. No significant risk of “all cancers” was found with “ever use of hair dyes”. No significant association was found in cancer sites such as breast, lung, stomach, colorectal, and overall hematopoietic cancers.

A2 Epidemiological studies on cancer risk from occupational exposures in the polyurethane industry

Human exposure to 2,4-DAT and 2,6-DAT primarily comes from occupational exposure during the production and handling of polyurethane foam (Lewandowski *et al.*, 2005). The source of 2,4- and 2,6-DAT in this industry stems from the use of the carcinogen toluene diisocyanate (TDI) during manufacturing of polyurethane products (Lind *et al.*, 1996). 2,4- and 2,6-DAT are synthesis byproducts of the production of polyurethane from 2,4- and 2,6-TDI, as well as polyurethane foam degradation products (Batich *et al.*, 1989; Luu and White, 1993). Three cohort studies (Sweden, UK, and US) and two follow-up studies were identified in the literature and are summarized here.

- Swedish studies:
    - Hagmar *et al.* (1993): This Swedish cohort study included 4,154 workers that had been employed for at least one year from nine polyurethane foam manufacturing plants. The average duration of employment was 6.5 years and the average follow-up was 10.6 years. The TDI exposure period ranged from 1959 to 1987 with the highest exposure levels ranging between 0.026 and 0.68 mg/m^3. No significantly increased risk of cancer mortality or incidence was found. The following increases of standardized incidence ratios (SIRs) were found but none was significant: SIR = 1.66 (95% CI 0.61-3.61) for rectal cancer, SIR = 1.53 (95% CI 0.42-3.91) for NHL, and SIR = 1.27 (95% CI 0.26-3.70) for leukemia.
    - Mikoczy *et al.* (2004): This is an update of the Swedish cohort by Hagmar *et al.* (1993), with 11 more years of follow-up and a total of 83,023 person-years observation. There was a significantly increased risk of lung cancer mortality among female employees, with a standardized mortality ratio (SMR) = 3.52 (95% CI 1.69-6.48). A nested case-control study of the association between polyurethane dust and lung cancer in female workers did not result in any increased risk with an OR = 0.8 (95% CI 0.2-2.9).

- UK studies:
    - Sorahan and Pope (1993): This UK cohort study consisted of 8,266 male and female polyurethane production workers (employed for at least six months) from 1958-1979 in 11 factories located in England and Wales. Excess mortality was found in women for pancreatic cancer (SMR = 2.71, 95% CI 1.00-5.95) and lung cancer (SMR = 1.76, 95% CI 1.00-2.85). No other statistically significant associations were found for women or men.
    - Sorahan and Nichols (2002): This is an update of the UK polyurethane production worker cohort (Sorahan and Pope, 1993). Significantly elevated risks were found in mortality from leukemia in men (SMR = 5.07, 95% CI 1.38-12.98), and lung and bronchial cancer in women (SMR = 1.81, 95% CI 1.26-2.51). Significantly elevated incidences were found in women for lung (SIR = 1.99, 95% CI 1.35-2.82) and pharynx cancer (SIR = 5.00, 95% CI 1.03-14.61) based on data from cancer registrations (1971-1994). The increased risk of lung cancer in women was consistent with findings from the previous report in this cohort. No significant associations between lung cancer risk and duration of exposure to diisocyanates were identified.

- US study:
    - Schnorr *et al.* (1996): This is a mortality study on a polyurethane foam occupational cohort of 4,611 workers employed by US four plants for at least three months between the late 1950s and 1987. The mortality due to rectal cancer and NHL were elevated but not statistically significant: rectal cancer SMR = 2.78 (95% CI 0.57-8.13), NHL SMR = 1.54 (95% CI 0.42-3.95) for NHL.

A3 Epidemiology studies on cancer risk from exposure to polyurethane-covered breast implants

Polyurethane-coated breast implants were removed from the US market by the manufacturer in 1991, after a study showed that the polyurethane can “degrade and release 2-toluene diamine (TDA) [2,4-DAT], a chemical known to cause cancer in animals, under conditions of high temperature and alkalinity (high pH)” (FDA, 2013). Three epidemiological studies on cancer risk and polyurethane-coated breast implants were identified in the literature and are summarized here.

- Brisson *et al.* (2006): This is a Canadian cohort study analysis of 24,558 women with bilateral cosmetic breast augmentation and 15,893 women with other cosmetic procedures between 1974 and 1989. Within the women with breast implants of any type, no association was found between polyurethane-coated breast implants and

breast cancer risk, with an RR = 1.51 (95% CI 0.86-2.65). However, within the women who received subglandular breast implants, there was a significant breast cancer risk with polyurethane-coated as compared to non-polyurethane coated breast implants, with an RR = 1.99 (95% CI 1.07-3.71). The RR in women with subglandular polyurethane-coated breast implants was highest during the first five years after surgery (RR = 7.01 [95% CI 1.18-41.71]), and decreased as time since surgery increased, with a RR = 1.71 (95% CI 0.58-4.99) 5-10 years after surgery, and an RR = 0.94 (95% CI 0.13-7.06) 10+ years after surgery.

- Lavigne *et al.* (2012): This is an extended follow-up analysis of a subset of the Canadian Breast Implant Cohort, with breast cancer patients with cosmetic breast implants (N=409) or other cosmetic surgery (N=444). Among the breast cancer patients with breast implants, there was a non-significant increased risk of higher cancer stage with polyurethane-coated breast implant compared to non-polyurethane coated implants, with an OR = 1.73 (95% CI 0.52-5.79).
- Pan *et al.* (2012): This is another analysis of the Canadian Breast Implant Cohort, with women who received either cosmetic breast implants (N=24,558) or other cosmetic surgery (N=15,893) between 1974 and 1989. Over 70% of the implant cohort was followed for over 20 years. Within the implant cohort, women who received polyurethane-coated implants had a higher incidence rate ratio (IRR) for breast cancer (albeit non-significant) compared to those receiving non-polyurethane coated implants: IRR=1.22 (95% CI 0.84-1.77).

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Appendix B. Microarray Data for 2,4-DAT and 2,6-DAT

Table B1. Expression levels of 54 genes from the livers of rats treated with 2,6-DAT or 2,4-DAT (Nakayama *et al.,* 2006)

Gene name	GenBank ID	Description	Biological process	2,4-DAT	2,6-DAT
Afar	CR457955	Aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)	Aflatoxin catabolism	2.55	0.84
Aldh1a1	CK222517	Aldehyde dehydrogenase family 1, member A1	Aldehyde metabolism	5.87	1.21
Hsd17b9	BC091114	Hydroxysteroid (17-beta) dehydrogenase 9	Androgen metabolism	0.77	1.05
Bax	U49729	Bcl2-associated X protein	Apoptosis	1.39	1.08
Tnfrsf6 (GenBank ID AI231531)	AI231531	Tumor necrosis factor receptor superfamily, member 6	Apoptosis	1.81	0.86
Tnfrsf6 (GenBank ID CV796105)	CV796105	Tumor necrosis factor receptor superfamily, member 6	Apoptosis	1.28	0.86
C7	AA800691	Complement component 7	Induction of apoptosis	1.16	0.78
Ephx1	AA849229	Epoxide hydrolase 1, microsomal	Aromatic compound catabolism	2.02	0.96
Ccng1 (GenBank ID AI009504)	AI009504	Cyclin G1	Cell cycle	2.23	1.18
Ccng1 (GenBank ID BI280715)	BI280715	Cyclin G1	Cell cycle	2.69	1.4
Cdkn1a	AI029091	Cyclin-dependent kinase inhibitor 1A	Cell cycle arrest	2.1	1.24
Acmsd	BF291117	2-Amino-3-carboxymuconate-6-semialdehyde decarboxylase	Cell growth	0.53	1.23
Mgmt	AI044880	O-6-methylguanine-DNA methyltransferase	DNA dealkylation	2.91	1.03
Apoa2	M28615	Apolipoprotein A-II	Glucose metabolism	0.65	0.89
Gria3	M36420	Glutamate receptor, ionotropic, AMPA3 (alpha 3)	Glutamate signaling	1.37	0.93

Gene name	GenBank ID	Description	Biological process	2,4-DAT	2,6-DAT
			pathway		
Olr37	NM_001000504	Olfactory receptor 37	G-protein coupled receptor protein signaling pathway	0.78	0.95
Wig1	BE105850	Wild-type p53-induced gene 1	Induction of apoptosis	1.14	1.04
Cyp4f5	BQ203240	Cytochrome P450 4F5	Inflammatory response	1.82	1.15
Apoc3	CD052182	Apolipoprotein C-III	Lipid catabolism	0.65	0.95
Acsl3	AI407883	Acyl-CoA synthetase long-chain family member 3	Lipid metabolism	1.37	0.77
Apoa4	BQ205741	Apolipoprotein A-IV	Lipid metabolism	0.44	0.87
Sth2	AI169780	Sulfotransferase family 2A, dehydroepiandrosterone (DHEA)-preferring, member 1	Lipid metabolism	0.57	1.14
Cryab	AA943860	Crystallin, alpha B	Muscle development	1.37	0.97
Cblc	BE111370	Casitas B-lineage lymphoma c	Negative regulation of epidermal growth factor receptor activity	1.56	0.74
B7h3	AA944582	B7 homlog 3	Negative regulation of T cell proliferation	2.33	0.85
Ddah1	CB776291	Dimethylarginine dimethylaminohydrolase 1	Nitric oxide biosynthesis	1.56	1.04
Fhit	NM_021774	Fragile histidine triad gene	Nucleotide metabolism	0.22	0.92
pgy1	AI179258	ATP-binding cassette, sub-family B, member 1	Response to drug	11.47	0.7
Gstm1	AW521280	Glutathione S-transferase, mu 1	Sensory perception	1.8	0.93
Sulf2	AI712364	Sulfatase 2	Sulfur metabolism	1.38	1.07

Gene name	GenBank ID	Description	Biological process	2,4-DAT	2,6-DAT
Il2ra	BF415214	Interleukin 2 receptor, alpha chain	T cell homeostasis	1.57	0.87
Pgcp	CR462586	Plasma glutamate carboxypeptidase	Tissue regeneration	0.69	0.98
Slc16a10	AI071792	Solute carrier family 16 (monocarboxylic acid transporters), member 10	Transport	0.87	1.5
Aldh1a4	CK222915	Aldehyde dehydrogenase family 1, subfamily A4	NI	2.53	0.92
Atp6v1d	AI104706	ATPase, H+ transporting, V1 subunit D	NI	1.94	1.04
Btg2	AW144492	B-cell translocation gene 2, anti-proliferative	NI	1.14	0.87
Cyp2c13	CK229453	Cytochrome P450 2c13	NI	1.91	0.5
LOC500590	BF546681	Similar to T-cell antigen 4-1BB precursor–mouse	NI	1.65	0.8
Isg20l1	AW534772	Similar to hypothetical protein FLJ12484 (predicted)	NI	1.66	1.18
No name	AI501208	Similar to thioether S-methyltransferase	NI	0.23	1.29
No name	BF550050	Transcribed locus	NI	1.34	0.97
no name (GenBank ID AI169630)	AI169630	Transcribed locus	NI	1.78	1
no name (GenBank ID BP471452)	BP471452	Sterile alpha motif domain containing 7 (predicted)	NI	2.16	1
No name (GenBank ID CF109340)	CF109340	Transcribed locus	NI	1.64	1.11
Nupr1	CR468250	Nuclear protein 1	NI	0.87	1.61
Pbsn	CA340306	Probasin	NI	5.46	1.22
Phlda3	CB730343	Pleckstrin homology-like domain, family A, member 3	NI	1.13	0.71
RGD1308114	NM_001014146	Similar to cDNA sequence AF397014	NI	0.25	0.95
RGD1308695	AI060285	Similar to RIKEN cDNA 1200016B10 (predicted)	NI	1.44	1.11
RGD1560717	DY312020	Similar to hypothetical protein DKFZp313N0621 (predicted)	NI	0.6	1.16

Gene name	GenBank ID	Description	Biological process	2,4-DAT	2,6-DAT
Rhobtb2	CO405068	Rho-related BTB domain containing 2	NI	4.15	1
Serpina7	M63991	Serine (or cysteine) peptidase inhibitor, clade A, member 7	NI	2.11	0.99
Spink3	NM_152936	Serine protease inhibitor, Kazal type 3	NI	0.57	1.06
Tacstd1	CR469413	Tumor-associated calcium signal transducer 1	NI	1.44	0.81

NI: Not identified by the authors

Appendix C. ToxCast Data for 2,3-DAT, 2,4-DAT, and 3,4-DAT

Table C1. Active ToxCast HTS assays for 2,3-DAT

Assay	Target Gene Symbol	Biological Process	Organism	Cells/Cell line	AC_{50} (µM)
NVS_NR_hPPARg	PPARG	receptor binding	human	cell-free	0.000339
BSK_BE3C_MMP1_up	MMP1	regulation of gene expression	human	primary bronchial epithelial cell	0.0139
BSK_hDFCGF_MCSF_down	CSF1	regulation of gene expression	human	primary foreskin fibroblast	0.766
BSK_CASM3C_MCP1_down	CCL2	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and coronary artery smooth muscle cells	0.975
ATG_Ahr_CIS_up	AHR	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	0.978
BSK_CASM3C_uPAR_down	PLAUR	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and coronary artery smooth muscle cells	1.4
BSK_4H_Eotaxin3_down	CCL26	regulation of gene expression	human	primary umbilical vein endothelium	2.43

Assay	Target Gene Symbol	Biological Process	Organism	Cells/Cell line	AC_{50} (µM)
BSK_CASM3C_IL6_down	IL6	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and coronary artery smooth muscle cells	3.16
ACEA_T47D_80hr_Negative	NA	cell proliferation	human	T47D, a human breast cancer cell line	4.08
BSK_CASM3C_MCSF_down	CSF1	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and coronary artery smooth muscle cells	4.41
BSK_SAg_IL8_up	CXCL8	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and peripheral blood mononuclear cells	4.45
NVS_GPCR_hAdoRA2a	ADORA2A	receptor binding	human	cell-free	4.96
NVS_NR_cAR	AR	receptor binding	Chimpanzee	cell-free	6.89
BSK_3C_IL8_up	CXCL8	regulation of gene expression	human	primary umbilical vein endothelium	7.26
NVS_ADME_hCYP1A2	CYP1A2	regulation of catalytic activity	human	cell-free	7.38
BSK_LPS_CD40_down	CD40	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and peripheral blood mononuclear cells	8.3

Assay	Target Gene Symbol	Biological Process	Organism	Cells/Cell line	AC_{50} (µM)
NVS_TR_hNET	SLC6A2	receptor binding	human	cell-free	8.3
APR_HepG2_CellCycleArrest_72h_dn	NA	cell cycle	human	HepG2, a human liver carcinoma cell line	8.54
APR_HepG2_CellLoss_72h_dn	NA	cell death	human	HepG2, a human liver carcinoma cell line	10.1
BSK_hDFCGF_Proliferation_down	NA	cell proliferation	human	primary foreskin fibroblast	10.3
BSK_LPS_PGE2_down	PTGER2	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and peripheral blood mononuclear cells	10.6
BSK_CASM3C_Thrombomodulin_up	THBD	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and coronary artery smooth muscle cells	11.2
NVS_GPCR_hAdoRA1	ADORA1	receptor binding	human	brain tissue-based cell-free	12.3
BSK_CASM3C_VCAM1_down	VCAM1	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and coronary artery smooth muscle cells	12.8
BSK_3C_Eselectin_down	SELE	regulation of gene expression	human	primary umbilical vein endothelium	12.9

Assay	Target Gene Symbol	Biological Process	Organism	Cells/Cell line	AC_{50} (µM)
BSK_KF3CT_ICAM1_down	ICAM1	regulation of gene expression	human	primary co-culture of keratinocytes and foreskin fibroblasts	13.6
BSK_CASM3C_HLADR_down	HLA-DRA	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and coronary artery smooth muscle cells	14.1
BSK_LPS_VCAM1_down	VCAM1	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and peripheral blood mononuclear cells	14.7
BSK_4H_VCAM1_down	VCAM1	regulation of gene expression	human	primary umbilical vein endothelium	15
NVS_GPCR_hDRD1	DRD1	receptor binding	human	cell-free	16.5
BSK_3C_VCAM1_down	VCAM1	regulation of gene expression	human	primary umbilical vein endothelium	17.8
BSK_LPS_MCSF_down	CSF1	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and peripheral blood mononuclear cells	18
ATG_NRF2_ARE_CIS_up	NFE2L2	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	18.2
NVS_GPCR_p5HT2C	HTR2C	receptor binding	wild boar	brain tissue-based cell-free	18.2

Assay	Target Gene Symbol	Biological Process	Organism	Cells/Cell line	AC_{50} (µM)
Tox21_ARE_BLA_agonist_ratio	NFE2L2	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	18.3
BSK_LPS_IL8_up	CXCL8	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and peripheral blood mononuclear cells	19.2
NVS_GPCR_hAdrb2	ADRB2	receptor binding	human	cell-free	19.2
BSK_BE3C_PAI1_down	SERPINE1	regulation of gene expression	human	primary bronchial epithelial cell	20.5
BSK_3C_HLADR_down	HLA-DRA	regulation of gene expression	human	primary umbilical vein endothelium	20.6
NVS_ENZ_hDUSP3	DUSP3	regulation of catalytic activity	human	cell-free	21.3
NVS_ENZ_hPTPRC	PTPRC	regulation of catalytic activity	human	cell-free	21.5
NVS_ENZ_hES	BCHE	regulation of catalytic activity	human	cell-free	25
ATG_AP_1_CIS_up	JUN	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	25.8
APR_HepG2_MitoticArrest_72h_up	NA	cell cycle	human	HepG2, a human liver carcinoma cell line	26.1
NVS_ENZ_hGSK3b	GSK3B	regulation of catalytic activity	human	cell-free	29.2

Assay	Target Gene Symbol	Biological Process	Organism	Cells/Cell line	AC_{50} (µM)
Tox21_MitochondrialToxicity_ratio	NA	mitochondrial depolarization	human	HepG2, a human liver carcinoma cell line	30.1
ATG_CMV_CIS_up	NA	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	33.4
BSK_KF3CT_MMP9_down	MMP9	regulation of gene expression	human	primary co-culture of keratinocytes and foreskin fibroblasts	37.9
APR_HepG2_OxidativeStress_24h_up	NA	oxidative phosphorylation	human	HepG2, a human liver carcinoma cell line	41.3
ATG_p53_CIS_up	TP53	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	42.8
ATG_HIF1a_CIS_up	HIF1A	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	45.8
Tox21_AhR	AHR	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	48.9
ATG_RXRb_TRANS_up	RXRB	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	50
ATG_PPRE_CIS_up	PPARA	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	55.9

Assay	Target Gene Symbol	Biological Process	Organism	Cells/Cell line	AC_{50} (µM)
APR_HepG2_OxidativeStress_72h_up	NA	oxidative phosphorylation	human	HepG2, a human liver carcinoma cell line	59.9
ATG_ERa_TRANS_up	ESR1	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	65.4
ATG_DR5_CIS_up	RARA	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	74.3
ATG_RORE_CIS_up	RORA	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	75.8
ATG_NFI_CIS_up	NFIA	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	81.7
ATG_EGR_CIS_up	EGR1	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	82.1
ATG_Myc_CIS_up	MYC	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	96.4
APR_HepG2_MitoMembPot_24h_dn	NA	mitochondrial depolarization	human	HepG2, a human liver carcinoma cell line	102
ATG_VDRE_CIS_up	VDR	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	105

Assay	Target Gene Symbol	Biological Process	Organism	Cells/Cell line	AC_{50} (µM)
APR_HepG2_p53Act_72h_up	TP53	cell death	human	HepG2, a human liver carcinoma cell line	107
ATG_MRE_CIS_up	MTF1	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	113
ATG_Pax6_CIS_up	PAX6	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	115
ATG_Xbp1_CIS_up	XBP1	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	125
ATG_CRE_CIS_up	CREB3	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	129
ATG_Oct_MLP_CIS_up	POU2F1	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	135
ATG_Sp1_CIS_up	SP1	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	164

Table C2. Active ToxCast assays for 2,4-DAT

Assay	Target Gene Symbol	Biological Process	Organism	Cells/Cell Line	AC_{50} (µM)
Tox21_AhR	AHR	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	4.28
ATG_Ahr_CIS_up	AHR	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	4.55
NVS_ENZ_rCNOS	Nos1	receptor binding	rat	brain tissue-based cell-free	6.78
BSK_CASM3C_Thrombomodulin_up	THBD	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and coronary artery smooth muscle cells	7.48
BSK_hDFCGF_Proliferation_down	NA	cell proliferation	human	primary foreskin fibroblast	15.2
NVS_ENZ_rabI2C	CKB	receptor binding	rabbit	brain tissue-based cell-free	20.8
NVS_ENZ_hPTPRC	PTPRC	regulation of catalytic activity	human	cell-free	32.2

Table C3. Active ToxCast HTS assays for 3,4-DAT

Assay	Target Gene Symbol	Biological Process	Organism	Cells/Cell Line	AC_{50} (µM)
BSK_3C_uPAR_up	PLAUR	regulation of gene expression	human	primary umbilical vein endothelium	0.68
BSK_3C_Proliferation_down	NA	cell proliferation	human	primary umbilical vein endothelium	1.49
Tox21_ERa_LUC_BG1_Agonist	ESR1	regulation of transcription factor activity	human	BG1, a human ovarian cancer cell line	1.88
BSK_4H_VEGFRII_up	KDR	regulation of gene expression	human	primary umbilical vein endothelium	3.09
BSK_4H_SRB_down	NA	cell death	human	primary umbilical vein endothelium	3.1
BSK_3C_IL8_up	CXCL8	regulation of gene expression	Human	primary umbilical vein endothelium	3.22
BSK_hDFCGF_MIG_down	CXCL9	regulation of gene expression	human	primary foreskin fibroblast	3.3
BSK_SAg_Eselectin_down	SELE	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and peripheral blood mononuclear cells	3.6
BSK_hDFCGF_IP10_down	CXCL10	regulation of gene expression	human	primary umbilical vein endothelium	5.01
BSK_3C_VCAM1_down	VCAM1	regulation of gene expression	human	primary umbilical vein endothelium	5.04
BSK_3C_Eselectin_down	SELE	regulation of gene expression	human	primary umbilical vein endothelium	5.27
BSK_SAg_CD40_down	CD40	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and peripheral blood mononuclear cells	5.53

Assay	Target Gene Symbol	Biological Process	Organism	Cells/Cell Line	AC_{50} (µM)
ACEA_T47D_80hr_Negative	NA	cell proliferation	human	T47D, a human breast cancer cell line	5.61
BSK_4H_VCAM1_down	VCAM1	regulation of gene expression	human	primary umbilical vein endothelium	6.16
BSK_3C_HLADR_down	HLA-DRA	regulation of gene expression	human	primary umbilical vein endothelium	6.18
BSK_3C_SRB_down	NA	cell death	human	primary umbilical vein endothelium	6.94
Tox21_HSE_BLA_agonist_ratio	HSF1	regulation of transcription factor activity	human	HeLa, a human cervical cancer cell line	7.24
ATG_NRF2_ARE_CIS_up	NFE2L2	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	8.04
BSK_LPS_VCAM1_down	VCAM1	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and peripheral blood mononuclear cells	8.23
BSK_LPS_MCSF_down	CSF1	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and peripheral blood mononuclear cells	8.67
BSK_hDFCGF_CollagenIII_down	COL3A1	regulation of gene expression	human	primary foreskin fibroblast	8.72
BSK_LPS_PGE2_down	PTGER2	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and peripheral blood mononuclear cells	8.91
NVS_ENZ_rabI2C	CKB	receptor binding	rabbit	brain tissue-based cell-free	8.98

Assay	Target Gene Symbol	Biological Process	Organism	Cells/Cell Line	AC_{50} (µM)
BSK_CASM3C_IL6_up	IL6	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and coronary artery smooth muscle cells	9.89
BSK_CASM3C_Thrombomodulin_up	THBD	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and coronary artery smooth muscle cells	10
BSK_KF3CT_MMP9_down	MMP9	regulation of gene expression	human	primary co-culture of keratinocytes and foreskin fibroblasts	10.3
BSK_CASM3C_VCAM1_down	VCAM1	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and coronary artery smooth muscle cells	10.8
BSK_CASM3C_Proliferation_down	NA	cell proliferation	human	primary co-culture of umbilical vein endothelium and coronary artery smooth muscle cells	11.1
BSK_4H_Eotaxin3_down	CCL26	regulation of gene expression	human	primary umbilical vein endothelium	11.8
BSK_hDFCGF_Proliferation_down	NA	cell proliferation	human	primary foreskin fibroblast	13
BSK_hDFCGF_MCSF_down	CSF1	regulation of gene expression	human	primary foreskin fibroblast	14.4
Tox21_ARE_BLA_agonist_ratio	NFE2L2	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	16.3
Tox21_AhR	AHR	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	17.8

Assay	Target Gene Symbol	Biological Process	Organism	Cells/Cell Line	AC_{50} (µM)
BSK_CASM3C_LDLR_up	LDLR	regulation of gene expression	human	primary co-culture of umbilical vein endothelium and coronary artery smooth muscle cells	19.6
Tox21_TR_LUC_GH3_Antagonist	THRB	regulation of transcription factor activity	rat	GH3, a rat pituitary tumor cell line	20
ATG_VDRE_CIS_up	VDR	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	20.9
NVS_TR_hNET	SLC6A2	receptor binding	human	cell-free	21.2
ATG_EGR_CIS_up	EGR1	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	25.6
ATG_Ahr_CIS_up	AHR	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	25.9
ATG_RORE_CIS_up	RORA	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	27.6
ATG_MRE_CIS_up	MTF1	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	29
ATG_AP_1_CIS_up	JUN	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	34.8
ATG_PPRE_CIS_up	PPARA	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	36.3

Assay	Target Gene Symbol	Biological Process	Organism	Cells/Cell Line	AC_{50} (µM)
NVS_ENZ_oCOX1	PTGS1	regulation of catalytic activity	sheep	testis tissue-based cell-free	37.1
ATG_CMV_CIS_up	NA	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	40.3
ATG_Oct_MLP_CIS_up	POU2F1	regulation of transcription factor activity	human	HepG2, a human liver carcinoma cell line	43.9
Tox21_ELG1_LUC_Agonist	ATAD5	regulation of transcription factor activity	human	HEK293T, a human embryonic kidney cell line	68.1
Tox21_ERa_BLA_Antagonist_ratio	ESR1	regulation of transcription factor activity	human	HEK293T, a human embryonic kidney cell line	68.5
Tox21_AR_LUC_MDAKB2_Agonist	AR	regulation of transcription factor activity	human	MDA-kb2, a human breast cell line	89.1

Table C4. Active ToxCast assays for both 2,3-DAT and 3,4-DAT

Assay	Target Gene Symbol	Target Protein / Biological Process	AC_{50} (µM) 2,3-DAT	AC_{50} (µM) 3,4-DAT
ATG_RORE_CIS_up	RORA, RORB, and RORC	Upregulation of the transcriptional activity at the human RAR-related orphan receptor element (RORE), in response to the activation of RORA, RORB, and RORC	75.8	27.6
BSK_hDFCGF_Proliferation_down	N/A	Downregulation of cell viability	10.3	13
ATG_CMV_CIS_up	N/A	Upregulation of CMV transcription activity (internal marker)	33.4	40.3
BSK_3C_HLADR_down	HLA-DRA	Downregulation of the HLA class II histocompatibility antigen, DR alpha chain protein expression	20.6	6.18
BSK_4H_Eotaxin3_down	CCL26	Downregulation of C-C motif chemokine 26 protein expression	2.43	11.8
BSK_hDFCGF_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1) protein expression	0.766	14.4
BSK_LPS_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1)	18	8.67
BSK_3C_Eselectin_down	SELE	Downregulation of Selectin E protein expression	12.9	5.27
BSK_KF3CT_MMP9_down	MMP9	Downregulation of the matrix metallopeptidase 9 (MMP9) protein expression	37.9	10.3
BSK_LPS_PGE2_down	PTGER2	Downregulation of the prostaglandin E receptor 2 (subtype EP2) (Ptger2) protein expression	10.6	8.91
BSK_3C_VCAM1_down	VCAM1	Downregulation of vascular cell adhesion molecule 1 (VCAM1) protein expression	17.8	5.04
BSK_4H_VCAM1_down	VCAM1	Downregulation of VCAM1 protein expression	15	6.16
BSK_CASM3C_VCAM1_down	VCAM1	Downregulation of VCAM1 protein expression	12.8	10.8

Assay	Target Gene Symbol	Target Protein / Biological Process	AC_{50} (µM) 2,3-DAT	AC_{50} (µM) 3,4-DAT
ATG_RORE_CIS_up	RORA, RORB, and RORC	Upregulation of the transcriptional activity at the human RAR-related orphan receptor element (RORE), in response to the activation of RORA, RORB, and RORC	75.8	27.6
BSK_hDFCGF_Proliferation_down	N/A	Downregulation of cell viability	10.3	13
ATG_CMV_CIS_up	N/A	Upregulation of CMV transcription activity (internal marker)	33.4	40.3
BSK_3C_HLADR_down	HLA-DRA	Downregulation of the HLA class II histocompatibility antigen, DR alpha chain protein expression	20.6	6.18
BSK_4H_Eotaxin3_down	CCL26	Downregulation of C-C motif chemokine 26 protein expression	2.43	11.8
BSK_hDFCGF_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1) protein expression	0.766	14.4
BSK_LPS_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1)	18	8.67
BSK_3C_Eselectin_down	SELE	Downregulation of Selectin E protein expression	12.9	5.27
BSK_KF3CT_MMP9_down	MMP9	Downregulation of the matrix metallopeptidase 9 (MMP9) protein expression	37.9	10.3
BSK_LPS_PGE2_down	PTGER2	Downregulation of the prostaglandin E receptor 2 (subtype EP2) (Ptger2) protein expression	10.6	8.91
BSK_3C_VCAM1_down	VCAM1	Downregulation of vascular cell adhesion molecule 1 (VCAM1) protein expression	17.8	5.04
BSK_4H_VCAM1_down	VCAM1	Downregulation of VCAM1 protein expression	15	6.16
BSK_LPS_VCAM1_down	VCAM1	Downregulation of VCAM1 protein expression	14.7	8.23
ACEA_T47D_80hr_Negative	N/A	Inhibition of cell growth	4.08	5.61

Assay	Target Gene Symbol	Target Protein / Biological Process	AC_{50} (µM) 2,3-DAT	AC_{50} (µM) 3,4-DAT
ATG_RORE_CIS_up	RORA, RORB, and RORC	Upregulation of the transcriptional activity at the human RAR-related orphan receptor element (RORE), in response to the activation of RORA, RORB, and RORC	75.8	27.6
BSK_hDFCGF_Proliferation_down	N/A	Downregulation of cell viability	10.3	13
ATG_CMV_CIS_up	N/A	Upregulation of CMV transcription activity (internal marker)	33.4	40.3
BSK_3C_HLADR_down	HLA-DRA	Downregulation of the HLA class II histocompatibility antigen, DR alpha chain protein expression	20.6	6.18
BSK_4H_Eotaxin3_down	CCL26	Downregulation of C-C motif chemokine 26 protein expression	2.43	11.8
BSK_hDFCGF_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1) protein expression	0.766	14.4
BSK_LPS_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1)	18	8.67
BSK_3C_Eselectin_down	SELE	Downregulation of Selectin E protein expression	12.9	5.27
BSK_KF3CT_MMP9_down	MMP9	Downregulation of the matrix metallopeptidase 9 (MMP9) protein expression	37.9	10.3
BSK_LPS_PGE2_down	PTGER2	Downregulation of the prostaglandin E receptor 2 (subtype EP2) (Ptger2) protein expression	10.6	8.91
BSK_3C_VCAM1_down	VCAM1	Downregulation of vascular cell adhesion molecule 1 (VCAM1) protein expression	17.8	5.04
BSK_4H_VCAM1_down	VCAM1	Downregulation of VCAM1 protein expression	15	6.16
NVS_TR_hNET	SLC6A2	Induction of transporter function of SLC6A2 (a neurotransmitter transporter)	8.3	21.2
BSK_3C_IL8_up	CXCL8	Upregulation of the Interleukin 8 (IL-8) protein expression	7.26	3.22

Assay	Target Gene Symbol	Target Protein / Biological Process	AC_{50} (µM) 2,3-DAT	AC_{50} (µM) 3,4-DAT
ATG_RORE_CIS_up	RORA, RORB, and RORC	Upregulation of the transcriptional activity at the human RAR-related orphan receptor element (RORE), in response to the activation of RORA, RORB, and RORC	75.8	27.6
BSK_hDFCGF_Proliferation_down	N/A	Downregulation of cell viability	10.3	13
ATG_CMV_CIS_up	N/A	Upregulation of CMV transcription activity (internal marker)	33.4	40.3
BSK_3C_HLADR_down	HLA-DRA	Downregulation of the HLA class II histocompatibility antigen, DR alpha chain protein expression	20.6	6.18
BSK_4H_Eotaxin3_down	CCL26	Downregulation of C-C motif chemokine 26 protein expression	2.43	11.8
BSK_hDFCGF_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1) protein expression	0.766	14.4
BSK_LPS_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1)	18	8.67
BSK_3C_Eselectin_down	SELE	Downregulation of Selectin E protein expression	12.9	5.27
BSK_KF3CT_MMP9_down	MMP9	Downregulation of the matrix metallopeptidase 9 (MMP9) protein expression	37.9	10.3
BSK_LPS_PGE2_down	PTGER2	Downregulation of the prostaglandin E receptor 2 (subtype EP2) (Ptger2) protein expression	10.6	8.91
BSK_3C_VCAM1_down	VCAM1	Downregulation of vascular cell adhesion molecule 1 (VCAM1) protein expression	17.8	5.04
BSK_4H_VCAM1_down	VCAM1	Downregulation of VCAM1 protein expression	15	6.16
ATG_PPRE_CIS_up	PPARA, PPARD, and PPARG	Upregulation of the transcriptional activity induced by the activation of peroxisome proliferator-activated receptors (PPAR alpha, delta, and gamma)	55.9	36.3

Assay	Target Gene Symbol	Target Protein / Biological Process	AC_{50} (µM) 2,3-DAT	AC_{50} (µM) 3,4-DAT
ATG_RORE_CIS_up	RORA, RORB, and RORC	Upregulation of the transcriptional activity at the human RAR-related orphan receptor element (RORE), in response to the activation of RORA, RORB, and RORC	75.8	27.6
BSK_hDFCGF_Proliferation_down	N/A	Downregulation of cell viability	10.3	13
ATG_CMV_CIS_up	N/A	Upregulation of CMV transcription activity (internal marker)	33.4	40.3
BSK_3C_HLADR_down	HLA-DRA	Downregulation of the HLA class II histocompatibility antigen, DR alpha chain protein expression	20.6	6.18
BSK_4H_Eotaxin3_down	CCL26	Downregulation of C-C motif chemokine 26 protein expression	2.43	11.8
BSK_hDFCGF_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1) protein expression	0.766	14.4
BSK_LPS_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1)	18	8.67
BSK_3C_Eselectin_down	SELE	Downregulation of Selectin E protein expression	12.9	5.27
BSK_KF3CT_MMP9_down	MMP9	Downregulation of the matrix metallopeptidase 9 (MMP9) protein expression	37.9	10.3
BSK_LPS_PGE2_down	PTGER2	Downregulation of the prostaglandin E receptor 2 (subtype EP2) (Ptger2) protein expression	10.6	8.91
BSK_3C_VCAM1_down	VCAM1	Downregulation of vascular cell adhesion molecule 1 (VCAM1) protein expression	17.8	5.04
BSK_4H_VCAM1_down	VCAM1	Downregulation of VCAM1 protein expression	15	6.16
ATG_AP_1_CIS_up	JUN	Upregulation of the transcriptional activity of AP-1, in response to activated Jun proto-oncogene	25.8	34.8

Assay	Target Gene Symbol	Target Protein / Biological Process	AC_{50} (µM) 2,3-DAT	AC_{50} (µM) 3,4-DAT
ATG_RORE_CIS_up	RORA, RORB, and RORC	Upregulation of the transcriptional activity at the human RAR-related orphan receptor element (RORE), in response to the activation of RORA, RORB, and RORC	75.8	27.6
BSK_hDFCGF_Proliferation_down	N/A	Downregulation of cell viability	10.3	13
ATG_CMV_CIS_up	N/A	Upregulation of CMV transcription activity (internal marker)	33.4	40.3
BSK_3C_HLADR_down	HLA-DRA	Downregulation of the HLA class II histocompatibility antigen, DR alpha chain protein expression	20.6	6.18
BSK_4H_Eotaxin3_down	CCL26	Downregulation of C-C motif chemokine 26 protein expression	2.43	11.8
BSK_hDFCGF_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1) protein expression	0.766	14.4
BSK_LPS_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1)	18	8.67
BSK_3C_Eselectin_down	SELE	Downregulation of Selectin E protein expression	12.9	5.27
BSK_KF3CT_MMP9_down	MMP9	Downregulation of the matrix metallopeptidase 9 (MMP9) protein expression	37.9	10.3
BSK_LPS_PGE2_down	PTGER2	Downregulation of the prostaglandin E receptor 2 (subtype EP2) (Ptger2) protein expression	10.6	8.91
BSK_3C_VCAM1_down	VCAM1	Downregulation of vascular cell adhesion molecule 1 (VCAM1) protein expression	17.8	5.04
BSK_4H_VCAM1_down	VCAM1	Downregulation of VCAM1 protein expression	15	6.16
ATG_EGR_CIS_up	EGR1	Upregulation of the transcriptional activity of human early growth response 1 (EGR1)	82.1	25.6

Assay	Target Gene Symbol	Target Protein / Biological Process	AC_{50} (µM) 2,3-DAT	AC_{50} (µM) 3,4-DAT
ATG_RORE_CIS_up	RORA, RORB, and RORC	Upregulation of the transcriptional activity at the human RAR-related orphan receptor element (RORE), in response to the activation of RORA, RORB, and RORC	75.8	27.6
BSK_hDFCGF_Proliferation_down	N/A	Downregulation of cell viability	10.3	13
ATG_CMV_CIS_up	N/A	Upregulation of CMV transcription activity (internal marker)	33.4	40.3
BSK_3C_HLADR_down	HLA-DRA	Downregulation of the HLA class II histocompatibility antigen, DR alpha chain protein expression	20.6	6.18
BSK_4H_Eotaxin3_down	CCL26	Downregulation of C-C motif chemokine 26 protein expression	2.43	11.8
BSK_hDFCGF_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1) protein expression	0.766	14.4
BSK_LPS_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1)	18	8.67
BSK_3C_Eselectin_down	SELE	Downregulation of Selectin E protein expression	12.9	5.27
BSK_KF3CT_MMP9_down	MMP9	Downregulation of the matrix metallopeptidase 9 (MMP9) protein expression	37.9	10.3
BSK_LPS_PGE2_down	PTGER2	Downregulation of the prostaglandin E receptor 2 (subtype EP2) (Ptger2) protein expression	10.6	8.91
BSK_3C_VCAM1_down	VCAM1	Downregulation of vascular cell adhesion molecule 1 (VCAM1) protein expression	17.8	5.04
BSK_4H_VCAM1_down	VCAM1	Downregulation of VCAM1 protein expression	15	6.16
ATG_MRE_CIS_up	MTF1	Upregulation of the transcriptional activity of metal-regulatory transcription factor 1 (Mtf1)	113	29

Assay	Target Gene Symbol	Target Protein / Biological Process	AC_{50} (µM) 2,3-DAT	AC_{50} (µM) 3,4-DAT
ATG_RORE_CIS_up	RORA, RORB, and RORC	Upregulation of the transcriptional activity at the human RAR-related orphan receptor element (RORE), in response to the activation of RORA, RORB, and RORC	75.8	27.6
BSK_hDFCGF_Proliferation_down	N/A	Downregulation of cell viability	10.3	13
ATG_CMV_CIS_up	N/A	Upregulation of CMV transcription activity (internal marker)	33.4	40.3
BSK_3C_HLADR_down	HLA-DRA	Downregulation of the HLA class II histocompatibility antigen, DR alpha chain protein expression	20.6	6.18
BSK_4H_Eotaxin3_down	CCL26	Downregulation of C-C motif chemokine 26 protein expression	2.43	11.8
BSK_hDFCGF_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1) protein expression	0.766	14.4
BSK_LPS_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1)	18	8.67
BSK_3C_Eselectin_down	SELE	Downregulation of Selectin E protein expression	12.9	5.27
BSK_KF3CT_MMP9_down	MMP9	Downregulation of the matrix metallopeptidase 9 (MMP9) protein expression	37.9	10.3
BSK_LPS_PGE2_down	PTGER2	Downregulation of the prostaglandin E receptor 2 (subtype EP2) (Ptger2) protein expression	10.6	8.91
BSK_3C_VCAM1_down	VCAM1	Downregulation of vascular cell adhesion molecule 1 (VCAM1) protein expression	17.8	5.04
BSK_4H_VCAM1_down	VCAM1	Downregulation of VCAM1 protein expression	15	6.16
ATG_NRF2_ARE_CIS_up	NFE2L2	Upregulation of the transcriptional activity of human nuclear factor, erythroid 2-like 2 (Nrf2)	18.2	8.04
Tox21_ARE_BLA_agonist_ratio	NFE2L2	Upregulation of the transcriptional activity of Nrf2	18.3	16.3

Assay	Target Gene Symbol	Target Protein / Biological Process	AC_{50} (µM) 2,3-DAT	AC_{50} (µM) 3,4-DAT
ATG_RORE_CIS_up	RORA, RORB, and RORC	Upregulation of the transcriptional activity at the human RAR-related orphan receptor element (RORE), in response to the activation of RORA, RORB, and RORC	75.8	27.6
BSK_hDFCGF_Proliferation_down	N/A	Downregulation of cell viability	10.3	13
ATG_CMV_CIS_up	N/A	Upregulation of CMV transcription activity (internal marker)	33.4	40.3
BSK_3C_HLADR_down	HLA-DRA	Downregulation of the HLA class II histocompatibility antigen, DR alpha chain protein expression	20.6	6.18
BSK_4H_Eotaxin3_down	CCL26	Downregulation of C-C motif chemokine 26 protein expression	2.43	11.8
BSK_hDFCGF_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1) protein expression	0.766	14.4
BSK_LPS_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1)	18	8.67
BSK_3C_Eselectin_down	SELE	Downregulation of Selectin E protein expression	12.9	5.27
BSK_KF3CT_MMP9_down	MMP9	Downregulation of the matrix metallopeptidase 9 (MMP9) protein expression	37.9	10.3
BSK_LPS_PGE2_down	PTGER2	Downregulation of the prostaglandin E receptor 2 (subtype EP2) (Ptger2) protein expression	10.6	8.91
BSK_3C_VCAM1_down	VCAM1	Downregulation of vascular cell adhesion molecule 1 (VCAM1) protein expression	17.8	5.04
BSK_4H_VCAM1_down	VCAM1	Downregulation of VCAM1 protein expression	15	6.16
ATG_Oct_MLP_CIS_up	POU2F1	Upregulation of the transcriptional activity of human POU class 2 homeobox 1 (Pou2f1)	135	43.9

Assay	Target Gene Symbol	Target Protein / Biological Process	AC_{50} (µM) 2,3-DAT	AC_{50} (µM) 3,4-DAT
ATG_RORE_CIS_up	RORA, RORB, and RORC	Upregulation of the transcriptional activity at the human RAR-related orphan receptor element (RORE), in response to the activation of RORA, RORB, and RORC	75.8	27.6
BSK_hDFCGF_Proliferation_down	N/A	Downregulation of cell viability	10.3	13
ATG_CMV_CIS_up	N/A	Upregulation of CMV transcription activity (internal marker)	33.4	40.3
BSK_3C_HLADR_down	HLA-DRA	Downregulation of the HLA class II histocompatibility antigen, DR alpha chain protein expression	20.6	6.18
BSK_4H_Eotaxin3_down	CCL26	Downregulation of C-C motif chemokine 26 protein expression	2.43	11.8
BSK_hDFCGF_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1) protein expression	0.766	14.4
BSK_LPS_MCSF_down	CSF1	Downregulation of Colony stimulating factor 1 (CSF-1)	18	8.67
BSK_3C_Eselectin_down	SELE	Downregulation of Selectin E protein expression	12.9	5.27
BSK_KF3CT_MMP9_down	MMP9	Downregulation of the matrix metallopeptidase 9 (MMP9) protein expression	37.9	10.3
BSK_LPS_PGE2_down	PTGER2	Downregulation of the prostaglandin E receptor 2 (subtype EP2) (Ptger2) protein expression	10.6	8.91
BSK_3C_VCAM1_down	VCAM1	Downregulation of vascular cell adhesion molecule 1 (VCAM1) protein expression	17.8	5.04
BSK_4H_VCAM1_down	VCAM1	Downregulation of VCAM1 protein expression	15	6.16
ATG_VDRE_CIS_up	VDR	Upregulation of the transcriptional activity at the Vitamin D response element (VDRE)	105	20.9
BSK_CASM3C_Thrombomodulin_up	THBD	Upregulation of thrombomodulin protein expression	11.2	10

Appendix D. Quantitative Structure Activity Relationship (QSAR) Models

QSAR models may be rule-based expert systems or statistical-based (Milan *et al.*, 2011). Rule-based expert systems codify chemical fragments into structural alerts (SAs) that are responsible for the toxic effect. These models are either based on expert human knowledge or the use of data-mining methods applied to structure-specific datasets. Statistical-based models translate the properties of chemicals into molecular descriptors and use an algorithm to determine a statistical correlation with chemical toxicity (Bakhtyari *et al.*, 2013).

OEHHA obtained models in the public domain used for carcinogenicity prediction and developed predictions for the DATs using models that were consistent with the Organisation for Economic Co-operation and Development (OECD) and the International Conference on Harmonisation (ICH) guidelines for model selection, as follows:

- The ICH M7 guidelines specify that two complementary QSAR methodologies should be used to predict the outcome of a bacterial mutagenicity assay: one rule-based and the other statistical-based (ICH, 2013). OEHHA applied the same criterion to model selection for carcinogenicity.
- The OECD requirements for each QSAR model were: 1) a defined endpoint, 2) an unambiguous algorithm, 3) a defined applicability domain, and 4) appropriate measures of goodness-of-fit, robustness and predictivity (OECD, 2007).

The models chosen for the analysis were the following:

- VEGA (a platform developed through a collaboration between the US EPA and a number of institutions from Europe under the sponsorship of ANTARES Project, CALEIDOS Project, CAESAR Project, Ministero della Salute, and ORCHESTRA Project) that contains three models:
    - CAESAR – a statistical-based system that predicts carcinogenicity toward male and female rats and bacterial mutagenicity
    - ToxTree – a rule-based system that predicts carcinogenicity toward humans, merging a series of rules from laboratory studies on different species and bacterial mutagenicity
    - SarPy – a statistical-based model developed in Python to predict mutagenicity.
- Lazar, which was developed by a Swiss company (In silico toxicology GMBH, 2011) for predicting toxicological endpoints.

- QSAR Toolbox, which was developed by the Laboratory of Mathematical Chemistry with the scientific and financial assistance of OECD and the European Union. It groups chemicals into categories and fills gaps in toxicity data in order to assess the hazards of chemicals.

Rule-based models reflect human mechanistic knowledge, while statistical-based models are able to assess large amounts of data to detect relationships not yet discovered by human experts. Most models clearly define the endpoint, algorithm, and various measures for validity. Table D1 provides a comparison of the QSAR models. It is important for models to use carcinogenicity and mutagenicity databases with large chemical inventories that contain data from tests done on more than one species or in more than one system. Internal and external validation of the datasets helps to ensure that the database is adequate for the model. Additionally, an important requirement of the OECD principles is for models to have a clear definition of the applicability domain. The applicability domain is the "response and chemical structure space in which the model makes predictions with a given reliability" (Netzeva *et al.*, 2005). This parameter is particularly important because the applicability domain expresses the range of chemical structures for which the model is considered to be applicable, *i.e.*, the scope and limitations of the model (Netzeva *et al.*, 2005). Predictions are reliable only when the model's assumptions are met; when the assumptions are violated, the prediction is unreliable (Jaworska *et al.*, 2005).

Statistical-based models, such as SarPy (VEGA NIC v1.0.8, 2013) and QSAR Toolbox (OECD QSAR Toolbox v. 3.2, 2013), commonly define the applicability domain using structural rules and/or descriptor variables in the dataset based on ranges, geometry, distances, or probability density distribution functions (Netzeva *et al.*, 2005). Another statistical-based model, CAESAR (VEGA NIC v1.0.8, 2013), evaluates the applicability domain with two methods. The general applicability domain is evaluated using a tool based on the range of chemical descriptors. Values that are predicted for chemicals outside the descriptor range are considered less reliable. To compensate for weaknesses in this general applicability domain approach, CAESAR also calculates a similarity score that compares the compound of interest to the six most similar chemicals in the dataset (Fjodorova *et al.*, 2010).

Other models, such as Lazar (In silico toxicology GMBH, 2011), provide a statistically-calculated confidence value, dependent on the "structural density" and similarity in activity of the neighborhood (Maunz and Helma, 2008). Decision tree approaches, such as ToxTree (VEGA NIC v1.0.8, 2013), define the applicability domain in terms of structural characteristics of the chemical classes to which they apply (Benigni *et al.*, 2008b). Calculations are based on the average Euclidean distance that the descriptors

of an unknown chemical are outside the range of those descriptors based on the training set (Netzeva *et al.*, 2005).

Table D1. Comparison of QSAR models

Model	Type of model (rule-based or statistical-based)	Basis for the applicability domain	Carcinogenicity prediction measure	Mutagenicity prediction measure
CAESAR	Statistical	Range of chemical descriptors and a similarity score	Carcinogen / non-carcinogen	Mutagen / non-mutagen
ToxTree	Rule	Structural characteristics of the chemical classes	Carcinogen / non-carcinogen	Mutagen / non-mutagen
SarPy	Statistical	Structural rules and/or descriptor variables	N/A[1]	Mutagen / non-mutagen
Lazar	Statistical	Confidence value dependent on the "structural density" and similarity in activity of the neighborhood	Carcinogen / non-carcinogen	Mutagen / non-mutagen
QSAR Toolbox	Statistical	Structural rules and/or descriptor variables	Positive / negative	Positive / negative

[1] N/A = not applicable; SarPy predicts for mutagenicity, not carcinogenicity

Descriptions of Models

VEGA

VEGA, a platform based on the CAESAR model, provides a qualitative prediction of carcinogenic potency toward male and female rats. The model was built on a Counter Propagation Artificial Neural Network (CP ANN) algorithm. Output of CP ANN contains two values, Positive and Non-Positive, which sum to one, representing the degree to which the neuron that the predicted chemical falls in belongs to the class of carcinogenic or non-carcinogenic compounds. The prediction is based on which value is higher. This model is based on the Distributed Structure-Searchable Toxicity (DSSTox) Public Database Network (US EPA, 2013a), which contains male and female rat data. It uses descriptors referring to topological characteristics, polarizability, and charge distribution (Fjodorova *et al.*, 2010). After the prediction has been made, specific SAs extracted from the ToxTree program are checked, and, if a match is found, a comment is given about the presence of a fragment that may be toxic (VEGA, 2013). CAESAR predicts mutagenicity by cascading two models. The first is a trained Support

Vector Machine classifier (a statistical model), and the second is a model that checks for SAs to remove false negatives (based on the Benigni-Bossa rulebase).

The other model in the VEGA platform uses the Benigni-Bossa rulebase for mutagenicity and carcinogenicity, which was developed as a module to the ToxTree software. This expert rule-based software applies rules based on human knowledge to identify SAs. Each SA represents a chemical class that causes toxic effects through one or more shared mechanisms of action. There are 33 SAs included in ToxTree; 28 are based on mechanisms of genotoxic carcinogenicity and five are based on non-genotoxic mechanisms. Compounds are flagged when at least one SA is recognized (Benigni *et al.*, 2008b).

A third model for mutagenicity included in VEGA is the SarPy model, a statistical model developed in Python (Istituto Mario Negri *et al.*, 2013). This is based on SAs that were selected by the program based on their occurrence in toxic or nontoxic compounds. It extracted 112 rules from the CAESAR model's dataset, and then used these to predict mutagenicity through the VEGA platform (Bakhtyari *et al.*, 2013).

Lazar

Lazar is an open-source software program that uses statistical methods to predict toxicological endpoints by analyzing structural fragments identified in an experimentally determined training set. It first identifies similar compounds in the training dataset, *i.e.*, "neighbors", based on structural, property, biological, or activity-specific similarities. It then generates a local prediction model based on the experimental activities of neighbors and uses the model to predict properties of the compound (Maunz *et al.*, 2013). It uses five databases for carcinogenicity based on the DSSTox database (DSSTox Carcinogenic Potency DBS MultiCellCall, DSSTox Carcinogenic Potency DBS Rat, DSSTox Carcinogenic Potency DBS SingleCellCall, DSSTox ISSCAN v3a Canc, DSSTox Carcinogenic Potency DBS Hamster, and DSSTox Carcinogenic Potency DBS Mouse) and two databases for mutagenicity (DSSTox Carcinogenic Potency DBS Mutagenicity and Kazius-Bursi Salmonella mutagenicity).

OECD QSAR Toolbox

QSAR Toolbox combines data and tools from many sources into a logical workflow. Users apply QSAR methodologies to group chemicals into categories based on physicochemical properties and human health hazards that are likely to be similar. After categories are assigned, data gap filling can be completed by three different methods: 1) read-across, 2) trend analysis, and 3) QSAR models. 1) *Read-across* predicts toxicity based on nearest neighbors in the chemical category compared by prediction descriptors (OECD, 2013). The descriptor values for the target chemical and the

category members are calculated using the rule "log K_{ow}," taking the weighted average value (OECD, 2009). Log K_{ow} is the octanol:water partition coefficient, a measure of hydrophobicity, which is important for transport and distribution, thus for predicting toxicity. Members of a category are often related by a trend, such as molecular mass, carbon chain length, or other physicochemical property, for a given endpoint. 2) *Trend analysis* estimates a value for an untested chemical from an increasing, decreasing, or constant trend for an effect within a category. 3) *QSAR models* allow the user to estimate missing values from a particular statistical model for a category (OECD, 2012).

Following data gap filling, a report is generated that allows the user to evaluate the strength of the read-across prediction. The model is capable of predicting carcinogenicity and mutagenicity for multiple species. Databases include the Carcinogenic Potency Database (CPDB) (Gold *et al.*, 2011), various ISS (Istituto Superiore di Sanità) Databases (Benigni *et al.*, 2008a), the Danish EPA database (Danish QSAR Group, 2004), Micronucleus and Genotoxicity OASIS, EXCHEM, and Toxicity Japan Ministry of Health, Labor and Welfare (LMC, 2013). An applicability domain is calculated based on the descriptor range for the dataset. The application alerts users when a chemical is out of the applicability domain or when there are not enough chemicals in the category to make a prediction. Additionally, a *p*-value for the prediction confidence is calculated, allowing the user to compare different profilers to determine the best prediction.

Strengths and weakness of QSAR models

Rule-based models (*e.g.*, ToxTree) are advantageous in that they are transparent and the explanations reflect mechanistic knowledge (Milan *et al.*, 2011). These models, however, have several limitations. Potentially toxic chemicals are flagged, but no conclusions about nontoxic chemicals are given. The list of toxic residues is incomplete and some chemical classes exert their toxic effects through complicated mechanisms that are difficult to code through SAs (Benigni *et al.*, 2008b). Additionally, most known carcinogenic residues codify genotoxic activity; non-genotoxic carcinogens are underrepresented. Statistical-based models, on the other hand, are advantageous in that they are able to detect relationships not yet discovered by human experts (Milan *et al.*, 2011). Models using simple rules, such as electronic properties of compounds, present another methodology to predict carcinogenic or mutagenic activity. While unlikely to be capable of identifying all active PAHs, they may be useful to flag potential compounds of concern.

A number of papers assess the accuracy, sensitivity and specificity of QSAR models using external datasets. Overall, statistical models have higher accuracy than rule-based methods for both mutagenicity (Bakhtyari *et al.*, 2013) and carcinogenicity (Milan

et al., 2011). Some models (*e.g.*, VEGA CAESAR) have higher sensitivity, while others (*e.g.*, Lazar) have higher specificity (Milan *et al.*, 2011). Often a more conservative model with higher sensitivity is preferred to avoid false negatives (Bakhtyari *et al.*, 2013). According to Bakhtyari *et al.* (2013), the accuracy of high-performing models (*e.g.*, VEGA CAESAR) is similar to the *in vitro* reproducibility of the Ames test.

VEGA provides transparency and allows the user to easily conduct an assessment and interpret the output. VEGA clearly defines the statistical methods used in its models, as well as the applicability domain. Lazar also clearly describes its model, but has a simpler algorithm and does not include as many datasets as VEGA. OECD QSAR Toolbox, on the other hand, requires extensive training and knowledge of the program in order to use it. It allows the user greater freedom in selecting parameters and lets the user see the chemicals used in comparisons. However, the multiple steps in the analysis lead to considerable variation in predictions made by different users. Additionally, the algorithms used in the OECD QSAR Toolbox model are not clearly described.

Appendix D References

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APPENDIX E. Parameters For Literature Searches on the Carcinogenicity of DATs

General searches of the literature on the carcinogenicity of DATs were conducted under contract by the University of California, Berkeley (Charleen Kubota, M.L.I.S.). The goal was 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 these chemicals. The search sought to specifically identify all literature relevant to the assessment of evidence on cancer.

Databases

The literature search utilized the following search platforms/database vendors:

- PubMed (National Library of Medicine)
- EMIC (National Library of Medicine)
- SciFinder®: CAS (Chemical Abstracts Service)
- TOXNET (National Library of Medicine): Toxicology Literature Online (TOXLINE), Genetic Toxicology Data Bank (GENE-TOX)
- Web of Knowledge: BIOSIS Previews®, Web of Science® (Thomson-Reuters, Inc.)

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])

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 listed above were then searched. The search strategies were tailored according to the search features unique to each database. 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 (RN) or the chemical name and its available synonyms. Sometimes other databases not listed here were searched as needed.

Additional focused searches were performed by OEHHA as needed. The search strategies are briefly described as follows:

Sections 3.1–3.3.5, Section 4

Focused searches were conducted for DATs. Relevant literature was also identified from citations in individual articles.

- Databases and other resources used: Google search engine, ChemSpider (Royal Society of Chemistry), ChemIDPlus (National Institutes of Health), MeSH (Medical Subject Headings) (National Library of Medicine), GENE-TOX, PubMed & PubChem BioAssay (National Library of Medicine), TOXLINE (National Library of Medicine).
- ChemSpider and ChemIDPlus were searched to gather synonyms, CAS RN, MeSH terms and Chemical Abstracts Service headings before searching bibliographic databases.
- PubMed search strategy: relevant subject terms were entered into the PubMed Search Builder to execute a PubMed search.
- The search strings applied to all databases listed above as: (“diaminotoluene” [MeSH] OR “toluenediamine” OR “methylphenylenediamine” OR “2,3-diaminotoluene” OR “2,4-diaminotoluene” OR “2,5-diaminotoluene” OR “2,6-diaminotoluene” OR “3,4-diaminotoluene” OR “3,5-diaminotoluene” OR “25376-45-8[RN]” OR “2687-25-4[RN]” OR” “95-80-7[RN]” OR “95-70-5[RN]” OR “823-40-5[RN]” OR “496-72-0[RN]” OR “26346-38-3[RN]” OR “26764-44-3[RN]” OR “108-71-4[RN]” OR “15481-70-6[RN]” OR “615-45-2[RN]” OR “615-50-9[RN]”) AND (“Neoplasms” [MeSH] OR “Cancer” [MeSH] OR “Mutation” [MeSH] AND “Toxicity” [MeSH] OR “Mechanism” [MeSH]).

Sections 3.3.6–3.3.7

- The DATs ToxCast/Tox21 data and assay information were found through iCSS Dashboard v0.5 (US EPA ToxCast Phase II data, http://actor.epa.gov/dashboard/) and PubChem BioAssay (National Library of Medicine)

- The toxicogenomic data on DATs were found through CTD (Comparative Toxicogenomics Database, http://ctdbase.org/) and PubMed searches.
- 23 papers related to associations between the target genes and cancer pathways were identified and selected using standard expert knowledge-based review practices.

Section 3.3.8:

- Structurally-related chemicals were identified using the following applications: ChemoTyper ((Molecular Networks GmbH and Altamira LLC, 2013)), OECD QSAR Toolbox ((OECD QSAR Toolbox v. 3.2, 2013)), and VEGA ((VEGA NIC v1.0.8, 201
- 3)).
- Databases and other resources used: Google search engine, ChemSpider (Royal Society of Chemistry), MeSH (Medical Subject Headings) (National Library of Medicine), PubMed & PubChem BioAssay (National Library of Medicine), TOXLINE (National Library of Medicine), iCSS Dashboard v0.5 (US EPA ToxCast Phase II data) and CTD (Comparative Toxicogenomics Database).
- The same search methods used in previous sections were used to identify data for structurally-similar chemicals.

In summary, 396 references, including government reports, peer-reviewed journal articles, and books, were identified through these search strategies. Among these, 300 were cited in this document.