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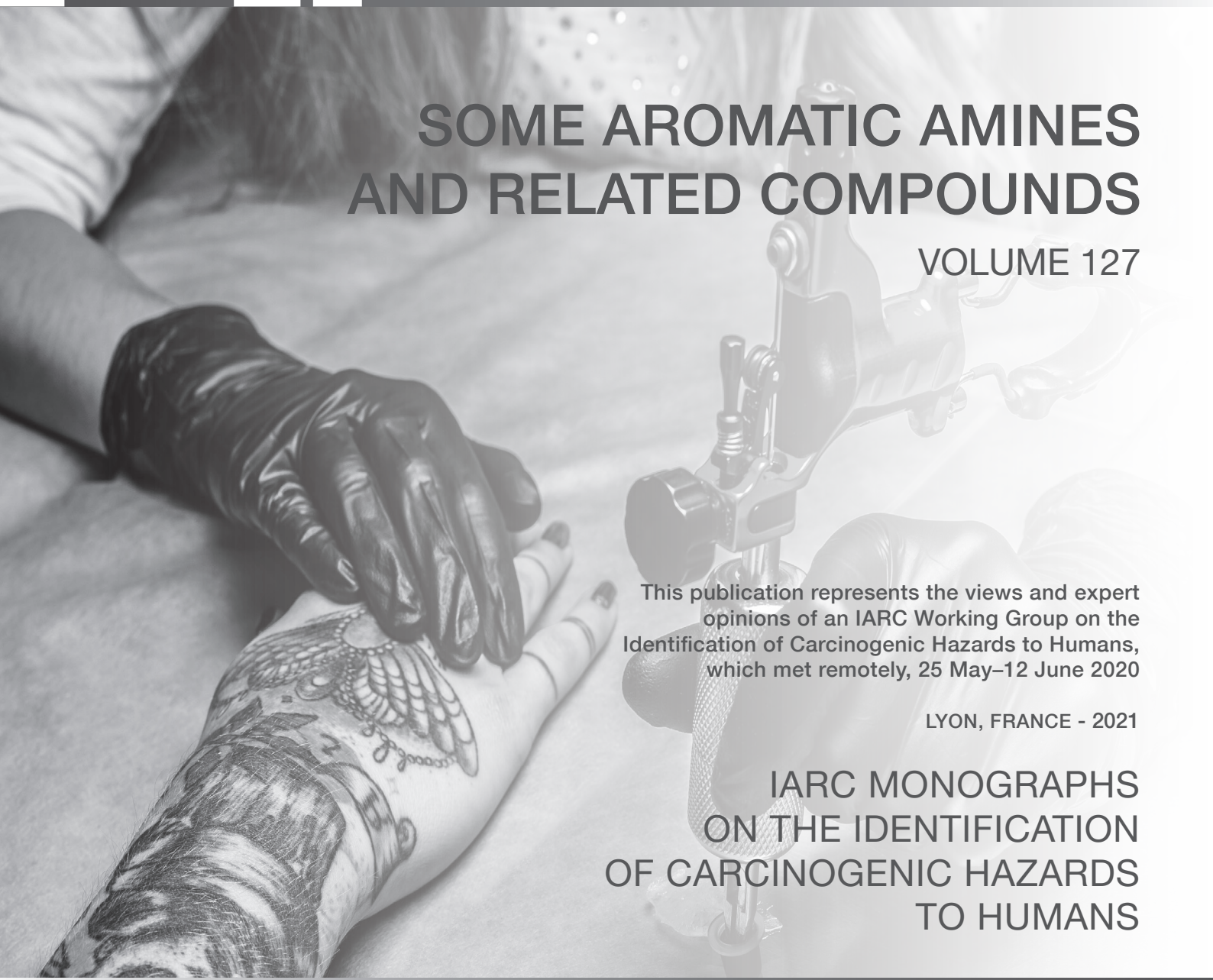


SOME AROMATIC AMINES AND RELATED COMPOUNDS

VOLUME 127

This publication represents the views and expert opinions of an IARC Working Group on the Identification of Carcinogenic Hazards to Humans, which met remotely, 25 May–12 June 2020

LYON, FRANCE - 2021

IARC MONOGRAPHS ON THE IDENTIFICATION OF CARCINOGENIC HAZARDS TO HUMANS

International Agency for Research on Cancer

World Health Organization

ORTHO-ANISIDINE AND *ORTHO*-ANISIDINE HYDROCHLORIDE

1. Exposure Characterization

1.1 Identification of the agent

1.1.1 Nomenclature

(a) ortho-*Anisidine*

Chem. Abstr. Serv. Reg. No.: 90-04-0

Chem. Abstr. Serv. name: benzenamine, 2-methoxy

EC No.: 201-963-1

IUPAC systematic name: 2-methoxyaniline

Synonyms: *o*-anisidine; 2-anisidine; 2-amino-anisole, 2-methoxyaniline; 1-amino-2-meth-oxybenzene; 2-methoxy-1-aminobenzene; *ortho*-methoxyaniline; 2-methoxybenzen-amine; *ortho*-methoxyphenylamine; 2-meth-oxyphenylamine; *o*-anisylamine.

(b) ortho-*Anisidine hydrochloride*

Chem. Abstr. Serv. Reg. No.: 134-29-2

Chem. Abstr. Serv. name: benzenamine, 2-methoxy-, hydrochloride

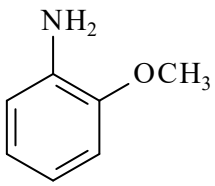
EC No.: 603-807-1

IUPAC systematic name: 2-methoxyaniline hydrochloride

Synonyms: *o*-anisidine.HCl; Fast Red BB Base; 2-aminoanisole hydrochloride; 2-meth-oxyaniline HCl; 2-anisidine hydrochloride; *o*-anisidine, hydrochloride; *o*-anisylamine hydrochloride.

1.1.2 Structural and molecular formulae, and relative molecular mass

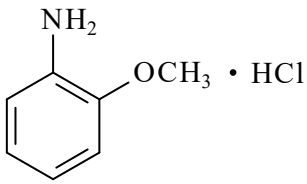
(a) ortho-*Anisidine*



Molecular formula: C_7H_9NO

Relative molecular mass: 123.15 (NCBI, 2020a).

(b) ortho-*Anisidine hydrochloride*



Molecular formula: $C_7H_{10}ClNO$

Relative molecular mass: 159.61 (NCBI, 2020b).

1.1.3 Chemical and physical properties of the pure substance

ortho-Anisidine is a basic compound and will undergo acid–base reactions. *ortho*-Anisidine and its hydrochloride salt will achieve a pH-dependent acid–base equilibrium in the body.

(a) ortho-*Anisidine*

Description: *ortho*-anisidine appears as clear, yellowish to reddish or brown liquid with an amine (fishy) odour (NCBI, 2020a)

Boiling point: 224 °C (NCBI, 2020a)

Melting point: 6.2 °C (NCBI, 2020a)

Density: 1.09 g/cm^3 at 20 °C (NCBI, 2020a)

Vapour density: 4.25 (air = 1) (NCBI, 2020a)

Vapour pressure: 10 Pa at 20 °C (ECHA, 2020)

Solubility: 14 g/L at 25 °C in water; miscible with ethanol, diethyl ether, acetone, and benzene (NTP, 2016)

Flash point: 107 °C, closed cup (NCBI, 2020a)

*Octanol/water partition coefficient (*P*)*: log K_{ow}, 1.18 (NTP, 2016)

Dissociation constant: pK_a, 4.53 (NTP, 2016)

Conversion factor: 1 ppm = 5.037 mg/m^3; 1 mg/m^3 = 0.199 ppm at 25 °C (European Commission, 2011).

(b) ortho-*Anisidine hydrochloride*

Description: *ortho*-anisidine hydrochloride is a grey-black crystalline solid or light grey powder (NCBI, 2020b)

Melting point: 225 °C (NTP, 2016)

Vapour pressure: 55 Pa at 25 °C (NTP, 2016)

Solubility: 10–50 g/L at 21 °C in water (NCBI, 2020b).

1.1.4 Technical grade and impurities

The purity of commercial *ortho*-anisidine is ≥ 99.0% and typically ≥ 99.4%. Possible impurities are aniline (≤ 0.4% w/w), *ortho*-chloranisole (≤ 0.2% w/w), *ortho*-chloraniline (≤ 0.4% w/w), and water (≤ 0.1% w/w) (European Chemicals Bureau, 2002).

1.2 Production and use

1.2.1 Production process

ortho-Anisidine is produced from *ortho*-nitroanisole (2-methoxy-nitrobenzene) by catalytic reduction with hydrogen under pressure in an inert liquid medium (European Commission, 2011). *ortho*-Anisidine hydrochloride is derived from *ortho*-anisidine (NCBI, 2020b).

1.2.2 Production volume

Production and imports of *ortho*-anisidine in the USA were in the range of 500 000 to less than 1 million pounds [230 to < 450 tonnes] in both 2015 and 2014, less than 1 million pounds [450 tonnes] in 2013, and 100 000–500 000 pounds [45–230 tonnes] in 2012 in the Chemical Data Reporting (CDR) database (US EPA, 2016). At least three companies in the USA manufacture *ortho*-anisidine. One company in Europe was listed as a Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) registrant, with *ortho*-anisidine listed only for use as an intermediate (ECHA, 2020).

It is estimated that less than 1000 tonnes of *ortho*-anisidine are produced annually within the European Union (European Commission, 2011). Information available indicated the presence of other manufacturer or supplier sites in India (Nandosal Chem Industries, 2020), China, and Japan (ChemNet, 2020). Historical data indicated that production plus imports of *ortho*-anisidine in the USA totalled 500 000

to 1 million pounds [230–450 tonnes] in 1986, 1990, and 2006; 1 million to 10 million pounds [450–4500 tonnes] in 1990 and 1998; and 10 000–500 000 pounds [4.5–230 tonnes] in 2002 (NTP, 2016). Additionally, information available in 1995 indicated that *ortho*-anisidine was produced in Armenia, China, France, Germany, India, Japan, Ukraine, and the United Kingdom (Chemical Information Services, 1995).

[The Working Group understood that *ortho*-anisidine hydrochloride is not produced in significant quantities commercially. For example, Sigma-Aldrich provides this product to early discovery researchers as part of a collection of unique chemicals (Merck, 2020).]

1.2.3 Uses

ortho-Anisidine is used as a chemical intermediate in the synthesis of azo pigments and dyes in consumer products, pharmaceuticals, and fragrances (for example, yellow/red azo pigments in hair dyes, tattoo ink, print ink, polymer dyes, and packaging foils, and in the manufacture of guaiacol and vanillin) (ECHA, 2011; Danish Environmental Protection Agency, 2012; NCBI, 2020a). About 90% of the dyes produced from *ortho*-anisidine are used in textiles, whereas the pigments are used mainly for printing paper and cardboard (European Commission, 2011). It is also used as a corrosion inhibitor and metal colourant in the automobile industry and in the production of steel storage tanks (IARC, 1999; Chaudhari et al., 2006; US EPA, 2019a). It is estimated that less than 850 tonnes of *ortho*-anisidine were used in the European Union in 1997 and this quantity declined in the early 2000s (European Commission, 2011).

ortho-Anisidine hydrochloride is used as a chemical intermediate to produce dyes, pigments, and pharmaceuticals, as a corrosion inhibitor, and as an antioxidant for polymercaptan resins (OEHHA, 1992).

1.3 Measurement and analysis

The presence of *ortho*-anisidine can be determined in water, soil, air, and solid waste samples using gas chromatography-mass spectrometry (GC-MS) with a quantitation limit of 10 µg/L in ground water (NEMI, 1998; EPA Method 8270D). The National Institute for Occupational Safety and Health (NIOSH) applies a method based on high-performance liquid chromatography with ultraviolet detection for the determination of *ortho*-anisidine in air samples, with a detection limit of 0.35 µg/sample (NIOSH, 2016; Method 2514 – Issue 3). Several GC-MS methods have been developed for the quantification of urinary *ortho*-anisidine: as reported, the limit of quantification was 0.05 µg/L [50 ng/L] (Kütting et al., 2009) and the limit of detection was between 7 ng/L (Mazumder et al., 2019) and 50 ng/L (Weiss & Angerer, 2002). Similarly, GC-MS was used for the measurement of *ortho*-anisidine and other aromatic amines in mainstream cigarette smoke, with a limit of detection of 7 pg/cigarette (Stabbert et al., 2003).

1.4 Occurrence and exposure

The primary routes of exposure to *ortho*-anisidine and *ortho*-anisidine hydrochloride are inhalation, skin absorption, ingestion, and eye contact (NCBI, 2020a).

1.4.1 Environmental occurrence

The United States Environmental Protection Agency (US EPA) Toxics Release Inventory (TRI) reported that 243 pounds [110 kg] of *ortho*-anisidine were released in the USA across all industries in 2018: 217 pounds [98 kg] to air emissions and 26 pounds [12 kg] to water discharges. There was little variation in the level of releases between 2012 and 2018 (US EPA, 2020). Monitoring data from 1993–1997 for German and Dutch rivers in most cases yielded *ortho*-anisidine levels

below or slightly above the limit of detection of 0.5 µg/L. Only peak concentrations in a highly polluted German river were equal to or above 5 µg/L (European Chemicals Bureau, 2002).

1.4.2 Occupational exposure

Regarding numbers of workers in publicly accessible exposure registries, the only information available to the Working Group was for the USA and Finland. The National Occupational Exposure Survey (conducted from 1981 to 1983) estimated that 705 workers classified as being employed in the chemicals and allied products industry were potentially exposed to *ortho*-anisidine and 1108 workers in the same industry were potentially exposed to *ortho*-anisidine hydrochloride (CDC, 2011). Six persons exposed to *ortho*-anisidine and its salts were recorded in the Finnish national register of workers exposed to carcinogenic substances and processes (Saalo et al., 2016). [The Working Group noted that information was sparse regarding numbers and global distribution of exposed workers.]

The following workplace 8 hour time-weighted average (TWA) concentrations were measured during production and processing of *ortho*-anisidine: production, 0.06–0.07 mg/m^3; long-term measurements of processing, 0.05–0.15 mg/m^3 (≥ 1 hour, shift average); and short-term measurements of processing, 0.05–0.09 mg/m^3 (< 1 hour). For the formulation of pigments (especially printing inks), the estimated exposure concentrations were between 0.07 and 28 ng/m^3. Dermal exposure concentrations for *ortho*-anisidine at the workplace were calculated using the EASE (Estimation and Assessment of Substance Exposure Physico-chemical properties) model. Significant exposure concentrations were derived only for the installation of gas compensation pipes, resulting in a maximum calculated body burden of 0.6 mg/kg body weight (bw) per day. The maximum calculated body burden for the formulation and use of *ortho*-anisidine–based printing inks was in the range of 6×10^{-5} to 1.5×10^{-3} mg/kg bw per day (European Chemicals Bureau, 2002).

1.4.3 Exposure of the general population

ortho-Anisidine has been detected in textiles, consumer products, cosmetics, and substances and products to which children may be exposed. In some cases, *ortho*-anisidine may be absent from the "ingredient list" for the product, but unexpectedly detected in product-testing studies (ECHA 2011; US EPA, 2019a). With the frequency of tattooing increasing while relevant regulations remain inconsistent, consumer use of tattoo inks is expected to be a growing source of population exposure to *ortho*-anisidine (ECHA 2011; European Commission, 2011; Danish Environmental Protection Agency, 2012; JRC, 2017)

ortho-Anisidine is also present in tobacco smoke. Mean concentrations of *ortho*-anisidine in mainstream tobacco smoke were reported to range from < 0.2 to 5.12 ng/cigarette (Stabbert et al., 2003). Health Canada reported an average of 4 ng/cigarette in mainstream tobacco smoke (Health Canada, 2009). The compound was detected in urine from 20 study participants in the general population without known exposure in Germany; the median was 0.22 µg/L, the range was < 0.05 to 4.2 µg/L, and 95% of participants had a detectable result (Weiss & Angerer, 2002). In a population-based cross-sectional study with more than 1000 volunteers in Bavaria, Germany, *ortho*-anisidine was detected at concentrations of up to 8.66 µg/L in urine samples, with median values of 0.23 µg/L. Although the sources of these exposures are not known, reported *ortho*-anisidine values in women were significantly higher in the urine of smokers than of non-smokers (Kütting et al., 2009) (see also Section 4.1.1(a)).

[The Working Group noted that these studies, together with the haemoglobin adduct study described in Section 4.2.1, provide an

inconsistent picture of the importance of tobacco smoking in contributing to levels of exposure to *ortho*-anisidine detected by biomonitoring.]

1.5 Regulations and guidelines

1.5.1 Exposure limits and guidelines

(a) US EPA

The reportable quantity under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) is 100 pounds [45 kg] for *ortho*-anisidine and is not indicated for *ortho*-anisidine hydrochloride (US EPA, 2019a). Releases of CERCLA hazardous substances, in quantities equal to or greater than their reportable quantity, are subject to reporting to the National Response Center under CERCLA and are also reportable under Emergency Planning and Community Right-To-Know Act (Section 313), or the US EPA's TRI (US EPA, 2019a). Emissions, transfers, and waste management data must be reported annually. Waste from the production of certain dyes, pigments, and food, drug, and cosmetic colourants – produced at a dye- or pigment-manufacturing site – is listed as US EPA hazardous waste K181. Under the K181 listing, *ortho*-anisidine is one of seven constituents whose presence in waste at a threshold amount (110 kg/year for *ortho*-anisidine) can serve as the basis for classifying the waste as hazardous (US EPA, 2003).

(b) OSHA

For *ortho*-anisidine, the current United States Occupational Safety and Health Administration (OSHA) permissible exposure limit (PEL) is 0.5 mg/m^3 of air as an 8-hour TWA. Absorption through the skin may be a significant source of exposure (NTP, 2016; OSHA, 2019).

(c) NIOSH

NIOSH lists an immediately dangerous to life and health (IDLH) concentration of 50 mg/m^3 for *ortho*-anisidine (NIOSH, 1994). It also lists a recommended exposure limit (REL) of 0.5 mg/m^3 TWA [skin]. NIOSH considers *ortho*-anisidine to be a potential occupational carcinogen as defined by the OSHA carcinogen policy (OSHA, 1990; NIOSH, 2018).

(d) European Chemicals Agency (ECHA)

ECHA has classified *ortho*-anisidine as carcinogenic (Category 1B), mutagenic (Category 2) and causing acute toxicity (Category 3). The use of *ortho*-anisidine is banned in any cosmetic products marketed for sale or use in the European Union (ECHA, 2020). It is on the candidate list of substances of very high concern (SVHC) for authorization – in accordance with Article 59(10) of the REACH Regulation – on the basis of carcinogenicity classification (ECHA, 2011). Workers who are aged < 18 years, pregnant, or breastfeeding, may not be exposed to *ortho*-anisidine. Employers are obliged to minimize other workers' exposure to *ortho*-anisidine as far as possible, and must arrange for medical surveillance of exposed workers (ECHA, 2020).

(e) Other international guidelines and limits

An occupational exposure limit (OEL), TWA, threshold limit value (TLV), and PEL of 0.5 mg/m^3 have also been adopted by many countries, including Argentina, Australia, Austria, Belgium, Bulgaria, Canada, China, Croatia, Denmark, France, Finland, Germany, Iceland, India, Indonesia, Ireland, Italy, Japan, Malaysia, Mexico, the Netherlands, New Zealand, Nicaragua, Norway, the Philippines, Poland, Portugal, the Republic of Korea, South Africa, Spain, Switzerland, and the United Arab Emirates. Romania has set an 8-hour limit value of 0.3 mg/m^3. In some countries, a short-term (15-minute average) limit value of 0.5–1.5 mg/m^3

has been adopted (European Chemicals Bureau, 2002; NICNAS, 2014; IFA, 2019). It is important to note that the United Kingdom Advisory Committee on Toxic Substances has expressed concern about the scientific basis for the derivation of the 0.5 mg/m^3 PEL and the adequacy of health protection it provides. This value was omitted from its 2005 OEL list and henceforth (HSE, 2020). The European Scientific Committee on Occupational Exposure Limits did not assign a TWA or short-term OEL for *ortho*-anisidine, citing insufficiency of evidence (European Commission, 2011). The California Environmental Protection Agency lists no-significant-risk levels (NSRLs) of 5 and 7 µg/day for *ortho*-anisidine and *ortho*-anisidine hydrochloride, respectively (OEHHA, 1992).

1.5.2 Reference values for biological monitoring of exposure

No reference values were available to the Working Group.

2. Cancer in Humans

Case reports

Nakano et al. (2018) reported data on 10 cases of cancer of the bladder in male Japanese workers exposed primarily to *ortho*-toluidine and employed at two plants producing organic dye and pigment intermediates. Of these 10 cases, 3 cases were also exposed to *ortho*-anisidine. Surrogate levels of exposure to six aromatic amines were calculated based on number of years and proportion of time spent on each of four production processes (preparation and reaction by mixing *ortho*-toluidine and diketene in organic solvent; filtering and rinsing the product with organic solvent; drying and packing the product; and distillation of waste organic solvent) each month. Two of the three *ortho*-anisidine-exposed cases were tobacco smokers. All 10 affected workers were hired between 1987 and 1997 and had been primarily engaged in drying and packing the product made from *ortho*-toluidine. Mean age at diagnosis for all cases of bladder cancer combined was 56 years (range, 41–71 years). [The Working Group considered that this study was not informative since all 3 *ortho*-anisidine-exposed cases were in workers co-exposed to *ortho*-toluidine and 2 of these cases were also in tobacco smokers; *ortho*-toluidine and tobacco smoking are both *carcinogenic to humans* (IARC Group 1) with *sufficient evidence* in humans for bladder cancer.]

3. Cancer in Experimental Animals

See Table 3.1.

3.1 Mouse

Oral administration (feed)

Groups of 55 male and 55 female B6C3F$_1$ mice (age, 41 days) were given feed containing *ortho*-anisidine hydrochloride (purity, > 99%) at a concentration of 0 (controls), 2500, or 5000 mg/kg, 7 days per week, for 103 weeks (105 weeks for the controls), followed by an additional observation period of 1–2 weeks (males at the lower dose) or 2 weeks (NCI, 1978). There was a slight (non-significant) dose-related positive trend in mortality among male and female mice. Numbers surviving to the end of the study were 44/55 (control group), 43/55 (lower dose), and 43/55 (higher dose) for males; and 44/55, 38/55, and 42/55, respectively, for females [sufficient numbers of mice of each sex were at risk of developing tumours]. There was a significant dose-related decrease in mean body weight in treated male and female mice compared with controls over the course of the

Table 3.1 Studies of carcinogenicity with *ortho*-anisidine and *ortho*-anisidine hydrochloride in mice and rats

Study design Species, strain (sex) Age at start Duration Reference	Route Agent, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (M) 41 days 104–105 wk NCI (1978)	Oral *ortho*-Anisidine hydrochloride, > 99% Feed 0, 2500, 5000 mg/kg, 7 days/wk, 103 wk 55, 55, 55 44, 43, 43	*Urinary bladder* Transitional cell papilloma 0/48, 2/55, 7/53* Transitional cell carcinoma 0/48, 0/55, 15/53* Transitional cell papilloma or carcinoma (combined) 0/48, 2/55, 22/53*	[$P = 0.010$ (Cochran–Armitage trend test); *$P < 0.02$ (Fisher exact test)] $P < 0.001$ (Cochran–Armitage trend test); *$P < 0.001$ (Fisher exact test) $P < 0.001$ (Cochran–Armitage trend test); *$P < 0.001$ (Fisher exact test)	Principal strengths: adequate number of mice used, randomly allocated in groups; adequate duration of exposure and observation and schedule of exposure; use of males and females. Controls put on study 3 wk earlier than treated groups. Statistical analyses were performed on mice that survived at least 52 wk, unless a tumour was observed before wk 52: comparisons were based on mice that survived at least as long as the mouse in which the tumour was found.
Full carcinogenicity Mouse, B6C3F$_1$ (F) 41 days 105 wk NCI (1978)	Oral *ortho*-Anisidine hydrochloride, > 99% Feed 0, 2500, 5000 mg/kg, 7 days/wk, for 103 wk 55, 55, 55 44, 38, 42	*Urinary bladder* Transitional cell carcinoma 0/50, 0/51, 18/50* Transitional cell papilloma or carcinoma (combined) 0/50, 1/51, 22/50*	$P < 0.001$ (Cochran–Armitage trend test); *$P < 0.001$ (Fisher exact test) $P < 0.001$ (Cochran–Armitage trend test); *$P < 0.001$ (Fisher exact test)	Principal strengths: adequate number of mice used, randomly allocated in groups; adequate duration of exposure and observation and schedule of exposure; use of males and females. Controls put on study 3 wk earlier than treated groups. Statistical analyses were performed on mice that survived at least 52 wk, unless a tumour was observed before wk 52: comparisons were based on mice that survived at least as long as the mouse in which the tumour was found.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Rat, F344 (M) 41 days 88 wk (high dose) or 104–106 wk NCI (1978)	Oral *ortho*-anisidine hydrochloride, > 99% Feed 0, 5000, 10 000 mg/kg, 7 days/wk, for 106 wk (control), 103 wk (low dose) or 88 wk (high dose) 55, 55, 55 39, 7, 0	*Urinary bladder* Transitional cell carcinoma 0/51, 50/54*, 51/52*	$P < 0.001$ (Cochran–Armitage trend test); *$P < 0.001$ (Fisher exact test)	Principal strengths: adequate number of rats used, randomly allocated in groups; adequate duration of exposure and observation and schedule of exposure; use of males and females. Positive dose-related trend in mortality. Controls put on study 3 wk earlier than treated groups. Statistical analyses were performed on rats that survived at least 52 wk, unless a tumour was observed before wk 52: comparisons were based on rats that survived at least as long as the rat in which the tumour was found. In the historical control data, the incidence of thyroid follicular cell tumours in male rats was 3/250 (1.2%).
		Transitional cell papilloma or carcinoma (combined) 0/51, 52/54*, 52/52*	$P < 0.001$ (Cochran–Armitage trend test); *$P < 0.001$ (Fisher exact test)	
		Renal pelvis: transitional cell carcinoma 0/53, 3/55, 7/53*	$P = 0.005$ (Cochran–Armitage trend test); *$P = 0.006$ (Fisher exact test)	
		Thyroid Follicular cell adenoma, cystadenoma, or papillary cystadenoma (combined) 0/53, 4/40*, 4/40*	$P = 0.030$ (Cochran–Armitage trend test); *$P = 0.031$ (Fisher exact test)	
		Follicular cell carcinoma or papillary cystadenocarcinoma (combined) 0/53, 3/40 (7.5%), 2/40 (5.0%)	NS	
		Follicular cell carcinoma, adenoma, cystadenoma, papillary cystadenoma, or papillary cystadenocarcinoma (combined) 0/53, 7/40 (17.5%)*, 6/40 (15.0%)**	$P = 0.009$ (Cochran–Armitage trend test); *$P = 0.002$ (Fisher exact test); **$P = 0.005$ (Fisher exact test)	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent, purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Incidence of tumours	Significance	Comments
Full carcinogenicity Rat, F344 (F) 41 days 83 wk (high dose) or 103–107 wk NCI (1978)	Oral *ortho*-Anisidine hydrochloride, > 99% Feed 0, 5000, 10 000 mg/kg, 7 days/wk, for 107 wk (control), 103 wk (low dose) or 83 wk (high dose) 55, 55, 55 36, 0, 0	*Urinary bladder* Transitional cell papilloma 0/49, 5/49*, 0/51 Transitional cell carcinoma 0/49, 41/49*, 50/51* Transitional cell papilloma or carcinoma (combined) 0/49, 46/49*, 50/51*	 *P = 0.028 (Fisher exact test) P < 0.001 (Cochran–Armitage trend test); *P < 0.001 (Fisher exact test) P < 0.001 (Cochran–Armitage trend test); *P < 0.001 (Fisher exact test)	Principal strengths: adequate number of rats used, randomly allocated in groups; adequate duration of exposure and observation and schedule of exposure; use of males and females. Positive dose-related trend in mortality. Controls put on study 3 wk earlier than treated groups. Statistical analyses were performed on rats that survived at least 52 wk, unless a tumour was observed before wk 52: comparisons were based on rats that survived at least as long as the rat in which the tumour was found.
Initiation–promotion (tested as promoter) Rat, F344 (M) 6 wk 36 wk Ono et al. (1992)	Oral *ortho*-Anisidine, NR Feed 0 (control), 1700 mg/kg (for 2 wk) then 425 mg/kg (for 30 wk) 0.05% *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in the drinking-water for 4 wk, then control feed or *ortho*-anisidine 15, 16 13, 16	*Urinary bladder* Papilloma 0/13, 3/16 Carcinoma 0/13, 2/16 Papillary or nodular hyperplasia 2/13, 13/16*	 NS NS *P < 0.01 (Fisher exact test and Student *t*-test)	Principal strengths: adequate duration and schedule of exposure and duration of observation. Principal limitation: no untreated control group was available. No urinary bladder lesions were observed in a group of 10 male rats receiving *ortho*-anisidine only.

F, female; M, male; NR, not reported; NS, not significant; wk, week.

study. An anatomopathological investigation was performed, and full histopathological examination was carried out on major organs, including some regional lymph nodes.

In male and female mice, there was a significant positive trend ($P < 0.001$) and a significant increase ($P < 0.001$, at the higher dose) in the incidence of transitional cell carcinoma and of transitional cell papilloma or carcinoma (combined) of the urinary bladder, compared with untreated male and female controls. In male mice, there was also a significant positive trend [$P = 0.010$] and a significant increase [$P < 0.02$] in the incidence of transitional cell papilloma of the urinary bladder at the higher dose compared with untreated controls. There was a significant increase in the incidence of focal hyperplasia of the urinary bladder in male and female mice at the higher dose. [The Working Group considered local hyperplasia of the urinary bladder to be a pre-neoplastic lesion.]

[The Working Group noted the adequate number of animals used, the random allocation in groups, the use of males and females, the adequate duration of exposure and observation, and the adequate schedule of exposure.]

3.2 Rat

3.2.1 Oral administration (feed)

Groups of 55 male and 55 female Fischer 344 rats (age, 41 days) were given feed containing *ortho*-anisidine hydrochloride (purity, > 99%) at a concentration of 0 (controls), 5000, or 10 000 mg/kg, 7 days per week, for 106–107 weeks for the controls, for 103 weeks for groups of males and females at the lower dose, for 88 weeks for males at the higher dose, and for 83 weeks for females at the higher dose. For males at the lower dose, there was an additional observation period of 1 week (NCI, 1978). There was a dose-related positive trend in mortality that was significant in male and female rats compared with controls. Numbers surviving to the end of the study were 39/55 (control group), 7/55 (lower dose), and 0/55 (higher dose) for males; and 36/55, 0/55, and 0/55, respectively, for females; however, 49/55 (89%) of males at the higher dose and 44/55 (80%) of females at the higher dose were still alive at week 52. All 55 rats in the groups of males and females at the lower dose and all 55 rats in the control groups of males and females lived beyond week 52. [The Working Group considered that sufficient numbers of male and female rats were at risk of developing tumours.] There was a significant dose-related decrease in mean body weight in treated male and female rats compared with controls. An anatomopathological investigation was performed, and full histopathological examination was carried out on major organs, including some regional lymph nodes.

There was a significant positive trend ($P = 0.005$) and a significant increase ($P = 0.006$) in the incidence of transitional cell carcinoma of the kidney/pelvis [renal pelvis] in male rats at the higher dose compared with male untreated controls. There was a significant positive trend ($P < 0.001$) and significant increase ($P < 0.001$) in the incidence of transitional cell carcinoma, and of transitional cell papilloma or carcinoma (combined) of the urinary bladder in treated groups of male and female rats at the lower and higher dose compared with untreated controls. There was also a significant increase ($P = 0.028$) in the incidence of transitional cell papilloma of the urinary bladder in females at the lower dose. In males, there was a significant positive trend ($P = 0.030$) and significant increase ($P = 0.031$) in the incidence of follicular cell adenoma, cystadenoma, or papillary cystadenoma (combined) of the thyroid at the lower and higher dose. In males, there was also a significant positive trend ($P = 0.009$) and significant increase ($P \leq 0.005$, lower and higher dose) in the incidence of follicular cell carcinoma, adenoma, cystadenoma, papillary cystadenoma, or papillary cystadenocarcinoma (combined) of the thyroid; the

incidence was: controls, 0/53; lower dose, 7/40 (17.5%); and higher dose, 6/40 (15.0%), respectively. The incidence of thyroid follicular cell carcinoma or papillary cystadenocarcinoma (combined) in the treated groups – controls, 0/53; lower dose, 3/40 (7.5%); and higher dose, 2/40 (5.0%) – was not significantly increased, but was higher than the incidence of thyroid follicular cell tumours reported for historical controls, which was 3/250 (1.2%) (NCI, 1978). [The Working Group noted the adequate number of animals used, the random allocation in groups, the use of males and females, the adequate duration of exposure and observation, and the adequate schedule of exposure.]

[The Working Group noted that the aromatic amines *ortho*-toluidine and 2-naphthylamine, which are *carcinogenic to humans* (IARC Group 1), also caused malignant tumours of the urinary bladder when administered orally in rats, and that the aromatic amine 4-aminobiphenyl (IARC Group 1) caused malignant tumours of the urinary bladder when administered orally in mice as well as in dogs (IARC, 2012).]

3.2.2 Initiation–promotion

Two groups of 15–16 male Fischer 344 rats (age, 6 weeks) were given drinking-water containing 0.05% *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) for 4 weeks. They were then given feed containing *ortho*-anisidine [purity not reported] at a concentration of 0 or 1700 mg/kg for the first 2 weeks and 425 mg/kg thereafter for an additional 30 weeks. A third group of 10 rats received *ortho*-anisidine without prior administration of BBN (Ono et al., 1992). At experimental week 36, the rats were killed and urinary bladders were examined histologically. The incidence of papillary or nodular hyperplasia of the urinary bladder was significantly higher ($P < 0.01$) in the group treated with BBN plus *ortho*-anisidine than in the group treated with BBN alone, but there was no significant increase in the incidence of papilloma or carcinoma of the urinary bladder. No lesions of the urinary bladder were observed in the group exposed to *ortho*-anisidine alone. [The Working Group noted that the duration of exposure and observation, and the schedule of exposure were adequate. No untreated control group was available.]

3.3 Synthesis

In one study in male and female B6C3F$_1$ mice treated by oral administration (in feed), *ortho*-anisidine hydrochloride caused a significant increase, with a significant positive trend, in the incidence of transitional cell papilloma, transitional cell carcinoma, and transitional cell papilloma or carcinoma (combined) of the urinary bladder in males, and of transitional cell carcinoma and transitional cell papilloma or carcinoma (combined) of the urinary bladder in females (NCI, 1978).

In one study in male and female Fischer 344 rats treated by oral administration (in feed), *ortho*-anisidine hydrochloride caused a significant increase, with a significant positive trend, in the incidence of transitional cell carcinoma and transitional cell papilloma or carcinoma (combined) of the urinary bladder, transitional cell carcinoma of the renal pelvis, and of follicular cell adenoma, cystadenoma, or papillary cystadenoma (combined) and follicular cell carcinoma, adenoma, cystadenoma, papillary cystadenoma, or papillary cystadenocarcinoma (combined) of the thyroid in males. *ortho*-Anisidine hydrochloride also caused a significant increase, with a significant positive trend, in the incidence of transitional cell carcinoma, and transitional cell papilloma or carcinoma (combined) of the urinary bladder in females. In addition, there was a significant increase in the incidence of transitional cell papilloma of the urinary bladder in treated female rats (NCI, 1978).

In one initiation–promotion study in which *ortho*-anisidine was tested as a promoter in

Fischer 344 male rats, there was no significant increase in the incidence of papilloma or carcinoma of the urinary bladder (Ono et al., 1992).

4. Mechanistic Evidence

4.1 Absorption, distribution, metabolism, and excretion

4.1.1 Humans

(a) Exposed humans

ortho-Anisidine was detected in 95% of urine samples in a study of 20 participants without known exposure. The concentration was in the range of 0.05 to 4.2 µg/L, with a median value of 0.22 µg/L and a 95th percentile of 0.68 µg/L (Weiss & Angerer, 2002).

In a cross-sectional study, urinary *ortho*-anisidine was quantified in 1004 volunteers aged 3–84 years. *ortho*-Anisidine was detected in 90% of the population at a concentration range of 0.03–8.66 µg/L, with a median value of 0.23 µg/L and a 95th percentile of 1.12 µg/L (Kütting et al., 2009).

No data on absorption after occupational exposure to *ortho*-anisidine were available to the Working Group.

(b) Human hepatic microsomes

See Fig. 4.1.

Stiborová et al. (2005) reported that *ortho*-anisidine (0.1–0.5 mM) incubated with human hepatic microsomes in the presence of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) produced two metabolites, one of which was identified as *N*-(2-methoxyphenyl)hydroxylamine. In addition, Stiborová et al. (2005) demonstrated the involvement of human cytochrome P450 (CYP) enzymes in the *ortho*-anisidine oxidation, mainly CYP2E1 and CYP1A2. These findings were supported by the results of three independent assays: (i) the highly significant correlation found between the rate of chlorzoxazone 6-hydroxylation, a marker for CYP2E1, and the levels of *N*-(2-methoxyphenyl)hydroxylamine in human hepatic microsomes; (ii) the inhibition of *N*-(2-methoxyphenyl)hydroxylamine formation by diethyldithiocarbamate, an inhibitor of CYP2E1 in human hepatic microsomes; and (iii) the oxidation of *ortho*-anisidine by recombinant human CYP enzymes in Supersomes, demonstrating that the enzymes CYP1A2, followed by CYP2B6 and CYP2E1, are the most efficient enzymes catalysing the metabolism of *ortho*-anisidine (Stiborová et al., 2005).

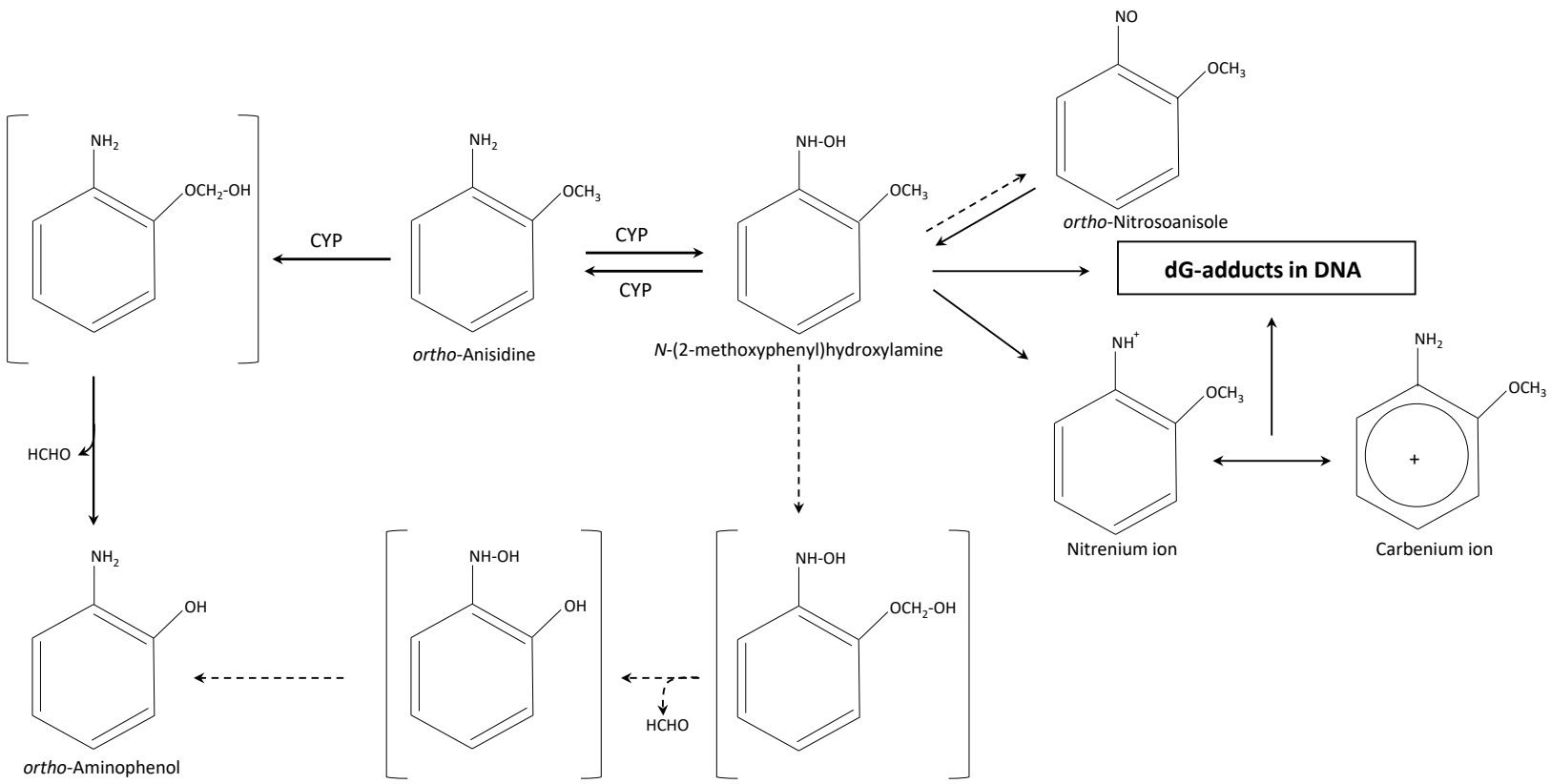
Naiman et al. (2011) reported that human CYP enzymes catalyse the further oxidative and reductive metabolism of *N*-(2-methoxyphenyl) hydroxylamine, the main metabolite of *ortho*-anisidine. Using human hepatic microsomes and human recombinant CYP enzymes incubated with *N*-(2-methoxyphenyl)hydroxylamine, the parent compound *ortho*-anisidine (major product), the isomer *ortho*-aminophenol, and two other metabolites were identified. In addition, the human CYP2E1, CYP3A4, and CYP2C enzymes were found to be important in catalysing the reduction of *N*-(2-methoxyphenyl) hydroxylamine to *ortho*-anisidine (Naiman et al., 2011).

4.1.2 Experimental systems

(a) In vivo

Sapota et al. (2003) reported on the tissue distribution, excretion, and metabolism of *ortho*-anisidine in male IMP:WIST rats given a single intraperitoneal dose of *ortho*-anisidine-ring-U-^{3}H (as a free base, dissolved in olive oil; 10 mg/kg bw). The blood plasma biphasic half-lives for fast and slow phases were about 1.5 hours and 80 hours, respectively, and the erythrocyte biphasic half-lives for fast and slow phases were about 1 hour and 116 hours,

Fig. 4.1 Pathways of *ortho*-anisidine metabolism



CYP, cytochrome P450; dG, deoxyguaonsine.
The compounds showed in square brackets were not detected under experimental conditions. The reactions shown in Fig. 4.1 that are more frequent, not as frequent, or infrequent are indicated by bold, normal, and dashed arrows, respectively.
Adapted from *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, Volume 726, issue 2, Naiman et al. (2011). Human cytochrome-P450 enzymes metabolize *N*-(2-methoxyphenyl)hydroxylamine, a metabolite of the carcinogens *o*-anisidine and *o*-nitroanisole, thereby dictating its genotoxicity, pages 160–188, copyright (2011), with permission from Elsevier.

respectively. *ortho*-Anisidine was widely distributed to tissues, with the highest levels found in the liver, kidneys, and muscle tissue. In all examined tissues except for kidney and fat, the highest concentration of radiolabel was found 12 hours after injection. Urine was the main route of excretion. Almost 72% was excreted during the first 72 hours, about 6% of this in the faeces (Sapota et al., 2003). The main urinary metabolites were: (i) *N*-acetyl-2-methoxyaniline (almost 97% of the total amount excreted in the urine); and (ii) *N*-acetyl-4-hydroxy-2-methoxyaniline (about 1.5% of the total amount excreted in the urine) (Sapota et al., 2003).

ortho-Anisidine induced methaemoglobinaemia in CBA mice and Alpk:APfSD rats treated by oral administration (Ashby et al., 1991), indicating that *ortho*-anisidine is distributed and N-oxidized in rodents.

(b) In vitro

See Fig. 4.1.

Oxidation of *ortho*-anisidine by peroxidases, including the mammalian peroxidases and prostaglandin H synthase (from ram seminal vesicles), has been reported (Thompson & Eling, 1991; Stiborová et al., 2001, 2002). The pattern of metabolites formed after incubation of *ortho*-anisidine (0.1–1.0 mM) with peroxidases was dependent on the concentration of *ortho*-anisidine, concentration of peroxidases, incubation time, and pH. Peroxidases oxidized *ortho*-anisidine to a diimine metabolite, which subsequently hydrolysed to form a quinone imine (Stiborová et al., 2002). [The Working Group noted that diimine and quinone imine are electrophilic species.]

Three studies identified the metabolites formed in incubations of *ortho*-anisidine (0.1–2.0 mM) with rat and rabbit hepatic microsomes (Rýdlová et al., 2005; Naiman et al., 2008a, b). These studies showed that *ortho*-anisidine is subject to redox cycling reactions. It is primarily oxidized to *N*-(2-methoxyphenyl)hydroxylamine (major metabolite), *ortho*-aminophenol, and an additional metabolite. *N*-(2-methoxyphenyl) hydroxylamine is either further oxidized to *ortho*-nitrosoanisole (2-methoxynitrosobenzene) or reduced to parental *ortho*-anisidine, which can be oxidized again to produce *ortho*-aminophenol (Rýdlová et al., 2005; Naiman et al., 2008a, b). Using purified rat and rabbit hepatic CYP enzymes, reconstituted with NADPH:P450 reductase, the ability of CYP1A1, 1A2, 2B2, 2B4, 2E1, and 3A6 to catalyse the oxidation of o*rtho*-anisidine was observed (Naiman et al., 2008b). The involvement of CYP2C, CYP2E1, CYP2D, and CYP2A, was observed in the reduction of *N*-(2-methoxyphenyl)hydroxylamine to *ortho*-anisidine (Naiman et al., 2010).

4.2 Evidence relevant to key characteristics of carcinogens

This section summarizes the evidence for the key characteristics of carcinogens (Smith et al., 2016), including whether *ortho*-anisidine is electrophilic or can be metabolically activated to an electrophile; is genotoxic; or alters cell proliferation, cell death, or nutrient supply. For the evaluation of other key characteristics of carcinogens, data were not available or considered insufficient.

4.2.1 Is electrophilic or can be metabolically activated to an electrophile

(a) Humans

(i) Exposed humans

Two studies detected the presence of haemoglobin adducts of *ortho*-anisidine in blood samples. Richter et al. (2001) detected haemoglobin adducts of *ortho*-anisidine, using capillary GC-MS, in blood samples of children from three different regions of southern Germany. The levels of *ortho*-anisidine–haemoglobin adducts were statistically significantly higher in children from an urban area (Munich, 1.3

million inhabitants) than in children from a less urban area (Augsburg, 250 000 inhabitants) and in children from a rural area (Eichstatt, 13 000 inhabitants). The regional differences in levels of *ortho*-anisidine–haemoglobin adducts were not related to tobacco exposure, since there were no major differences between children from smoking and non-smoking households (Richter et al., 2001). Haemoglobin adducts of *ortho*-anisidine, analysed by capillary GC-MS, were detected at similar levels in the blood of smoking and non-smoking pregnant women in Germany (Branner et al., 1998).

(ii) Human cells in vitro

No data from studies in human cells in vitro were available to the Working Group.

In two studies it was observed that *ortho*-anisidine is activated by human hepatic microsomes to form DNA adducts. Stiborová et al. (2005) used two techniques, [^{14}C]-labelled *ortho*-anisidine and ^{32}P-postlabelling, to show that after activation by human hepatic microsomes *ortho*-anisidine forms *N*-(2-methoxyphenyl) hydroxylamine and binds to DNA. Using the ^{32}P-postlabelling technique, Naiman et al. (2011) reported DNA-adduct formation induced by *N*-(2-methoxyphenyl)hydroxylamine, the main metabolite of *ortho*-anisidine, when incubated with human hepatic microsomes.

(b) Experimental systems

Covalent binding to DNA was undetectable in B6C3F$_1$ mouse bladder or liver cells in vivo, after a single oral dose of *ortho*-anisidine hydrochloride (Ashby et al., 1994; see Table 4.1). *ortho*-Anisidine–DNA adducts, detected by ^{32}P-postlabelling, were observed in the urinary bladder, liver, kidney, and spleen (but not in the lung, heart, or brain) of Wistar rats treated with more than one intraperitoneal dose of *ortho*-anisidine (Stiborová et al., 2005; Naiman et al., 2012; see Table 4.1). The highest total DNA-adduct levels were found in the urinary bladder. The level of adducts in the bladder declined with time, but 39% of the initial level of binding remained even after 36 weeks (Naiman et al., 2012). Covalent binding was much less persistent in the liver, kidney, and spleen. *N*-(Deoxyguanosin-8-yl)-2-methoxyaniline was the major DNA adduct formed by *ortho*-anisidine. [The Working Group noted that guanine is also the predominant deoxynucleotide target within DNA for covalent binding by other aromatic amines, such as 4-aminobiphenyl, which is classified in IARC Group 1 (IARC, 2012).] There was formation of DNA adducts, detected by ^{32}P-postlabelling, in the urinary bladder of Wistar rats exposed orally to *ortho*-anisidine hydrochloride for 4 weeks (Iatropoulos et al., 2015; see Table 4.1).

ortho-Anisidine (1 mM) underwent covalent binding to calf thymus DNA (Thompson & Eling, 1991). Metabolites of *ortho*-anisidine (diimine and quinone imine) were consistently more reactive with protein and glutathione than were metabolites of *para*-anisidine (Thompson & Eling, 1991). Two subsequent studies using [^{14}C]-labelled *ortho*-anisidine and ^{32}P-postlabelling assays observed that, after peroxidation to diimine and quinone imine, *ortho*-anisidine binds to calf thymus DNA in the presence of microsomes from ram seminal vesicles (Stiborová et al., 2001, 2002). Using [^{14}C]-labelled *ortho*-anisidine, Stiborová et al. (2002) observed substantial peroxidase-dependent covalent binding of *ortho*-anisidine to DNA, tRNA, and polydeoxynucleotides. Using the ^{32}P-postlabelling assay, and enzymatic digestion with three times higher concentrations of micrococcal nuclease and spleen phosphodiesterase than in the standard procedure, *ortho*-anisidine activated by peroxidases was bound to poly(dG)–poly(dC) and to a lesser extent to poly(dA), but binding to poly(dC) or poly(dT) was not detectable, suggesting specificity for purine adduct formation (Stiborová et al., 2002).

Table 4.1 Genetic and related effects of *ortho*-anisidine and *ortho*-anisidine hydrochloride in non-human mammals in vivo

End-point (assay)	Species, strain (sex)	Tissue	Results[a]	Agent, dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
DNA adducts (^{14}C-labelling assay)	Mouse, B6C3F$_1$ (F)	Bladder and liver	–	[^{14}C]-Labelled *ortho*-anisidine hydrochloride, 750 mg/kg bw	Oral, 1×		Ashby et al. (1994)
DNA adducts (^{32}P-post-labelling assay)	Mouse, B6C3F$_1$ (F)	Bladder and liver	–	*ortho*-Anisidine hydrochloride, 750 mg/kg bw	Oral, 1×		Ashby et al. (1994)
DNA adducts (^{32}P-post-labelling assay)	Rat, Wistar (M)	Bladder, liver, kidney and spleen	+	*ortho*-Anisidine, 0.15 mg/kg bw	Intraperitoneal, 1×/day for 5 days	DNA adducts detected using nuclease P1 version, but not with standard procedure	Stiborová et al. (2005)
DNA adducts (^{32}P-post-labelling assay)	Rat, Wistar (M)	Lung, heart and brain	–	*ortho*-Anisidine, 0.15 mg/kg bw	Intraperitoneal, 1×/day for 5 days		Stiborová et al. (2005)
DNA adducts (^{32}P-post-labelling assay)	Rat, Wistar (M)	Bladder, liver, kidney and spleen	+	*ortho*-Anisidine: day 1, 0.15 mg/kg bw; day 2, 0.18 mg/kg bw; and day 3, 0.2 mg/kg bw	Intraperitoneal, 1×/day for 3 days; total dose, 0.53 mg/kg bw	DNA adducts detected using nuclease P1 version and standard procedure under ATP-deficient conditions.	Naiman et al. (2012)
DNA adducts (^{32}P-post-labelling assay)	Rat, Wistar (M)	Bladder	+	*ortho*-Anisidine hydrochloride, 17 mg/kg bw	Oral, 3×/wk for 4 wk	DNA adducts detected using nuclease P1 version and HLB columns	Iatropoulos et al. (2015)
DNA strand breaks (alkaline comet assay)	Rat, Wistar (M)	Bladder	+	*ortho*-Anisidine hydrochloride, 17 mg/kg bw	Oral, 3×/wk for 4 wk		Iatropoulos et al. (2015)
DNA single-strand break (alkaline elution assay)	Rat, Sprague-Dawley (M)	Liver, thymus, and testis	–	*ortho*-Anisidine, 700 mg/kg bw	Oral, 1×		Ashby et al. (1991)
DNA single-strand break (automated alkaline elution system)	Rat, Wistar (M)	Liver, kidney, spleen and bladder	–	*ortho*-Anisidine, 500 mg/kg bw	Oral, 1×		Ashby et al. (1991)
DNA single-strand break (automated alkaline elution system)	Rat, Wistar (M)	Liver and bladder	–	*ortho*-Anisidine, 750 mg/kg bw	Intraperitoneal, 1×		Ashby et al. (1991)

Table 4.1 (continued)

End-point (assay)	Species, strain (sex)	Tissue	Results[a]	Agent, dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
DNA strand breaks (alkaline comet assay)	Rat, Wistar (M)	Stomach, colon, liver, kidney, bladder, lung, brain, and bone marrow	–	*ortho*-Anisidine, 1000 mg/kg bw	Oral, in olive oil; 1×; sampled after 0 h		Sekihashi et al. (2002)
DNA strand breaks (alkaline comet assay)	Rat, Wistar (M)	Colon, kidney, bladder, and lung	+	*ortho*-Anisidine, 1000 mg/kg bw	Oral, in olive oil; 1×; sampled after 3 h		Sekihashi et al. (2002)
DNA strand breaks (alkaline comet assay)	Rat, Wistar (M)	Stomach, liver, brain, and bone marrow	–	*ortho*-Anisidine, 1000 mg/kg bw	Oral, in olive oil; 1×; sampled after 3 h		Sekihashi et al. (2002)
DNA strand breaks (alkaline comet assay)	Rat, Wistar (M)	Stomach, colon, kidney, bladder, lung, and brain	+	*ortho*-Anisidine, 1000 mg/kg bw	Oral, in olive oil; 1×; sampled after 8 h		Sekihashi et al. (2002)
DNA strand breaks (alkaline comet assay)	Rat, Wistar (M)	Liver and bone marrow	–	*ortho*-Anisidine, 1000 mg/kg bw	Oral, in olive oil; 1×; sampled after 8 h		Sekihashi et al. (2002)
DNA strand breaks (alkaline comet assay)	Rat, Wistar (M)	Colon	+	*ortho*-Anisidine, 1000 mg/kg bw	Oral, in olive oil; 1×; sampled after 24 h		Sekihashi et al. (2002)
DNA strand breaks (alkaline comet assay)	Rat, Wistar (M)	Stomach, liver, kidney, bladder, lung, brain, and bone marrow	–	*ortho*-Anisidine, 1000 mg/kg bw	Oral, in olive oil; 1×; sampled after 24 h		Sekihashi et al. (2002)
DNA strand breaks (alkaline comet assay)	Rat, Sprague-Dawley (M) and (F)	Bladder	–	*ortho*-Anisidine, 700 mg/kg bw	Oral, 1×/day for 2 days		Wada et al. (2012)
DNA single-strand break (alkaline comet assay)	Rat, Crl:CD(SD) (M)	Liver		*ortho*-Anisidine, 600 mg/kg bw	Oral, 1×		Uno & Omori (2015)
DNA strand breaks (alkaline comet assay)	Rat, Sprague-Dawley (M)	Liver	±	*ortho*-Anisidine, 600 mg/kg bw	Oral, 1×/day, for 3 days	No dose–response relationship observed	Hobbs et al. (2015)
DNA strand breaks (alkaline comet assay)	Rat, Sprague-Dawley (M)	Stomach	–	*ortho*-Anisidine, 600 mg/kg bw	Oral, 1×/day, for 3 days		Hobbs et al. (2015)

Table 4.1 (continued)

End-point (assay)	Species, strain (sex)	Tissue	Results[a]	Agent, dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
DNA strand breaks (phosphorylated histone γ-H2AX)	Rat, Fischer/DuCrl-Crlj (M)	Bladder	+	*ortho*-Anisidine hydrochloride, 1% in feed (563.1 mg/kg bw)	Oral, for 2 days, 2 wk and 4 wk		Toyoda et al. (2019)
Unscheduled DNA synthesis	Rat, F344 (M)	Kidney	–	*ortho*-Anisidine, 500 mg/kg bw	Intraperitoneal, 1×/day		Tyson & Mirsalis (1985)
Unscheduled DNA synthesis	Rat, AP (M)	Liver	–	*ortho*-Anisidine, 1104 mg/kg bw	Oral, 1×		Ashby et al. (1991)
DNA strand breaks (alkaline comet assay)	Mouse, ddY (M)	Stomach, colon, liver, kidney, bladder, lung, brain and bone marrow	–	*ortho*-Anisidine, 690 mg/kg bw	Oral, in olive oil; 1×; sampled after 0 h		Sekihashi et al. (2002)
DNA strand breaks (alkaline comet assay)	Mouse, ddY (M)	Colon and bladder	+	*ortho*-Anisidine, 690 mg/kg bw	Oral, in olive oil; 1×; sampled after 3 h and 8 h		Sekihashi et al. (2002)
DNA strand breaks (alkaline comet assay)	Mouse, ddY (M)	Stomach, liver, kidney, lung, brain and bone marrow	–	*ortho*-Anisidine, 690 mg/kg bw	Oral, in olive oil; 1×; sampled after 3 h and 8 h		Sekihashi et al. (2002)
DNA strand breaks (alkaline comet assay)	Mouse, ddY (M)	Bladder	+	*ortho*-Anisidine, 690 mg/kg bw	Oral, in olive oil; 1×; sampled after 24 h		Sekihashi et al. (2002)
DNA strand breaks (alkaline comet assay)	Mouse, ddY (M)	Stomach, colon, liver, kidney, lung, brain and bone marrow	–	*ortho*-Anisidine, 690 mg/kg bw	Oral, in olive oil; 1×; sampled after 24 h		Sekihashi et al. (2002)
DNA strand breaks (alkaline comet assay)	Mouse, CD-1 (M)	Bladder	+	*ortho*-Anisidine, 690 mg/kg bw	Oral, 1×	Using modified comet assay; nuclei isolated by homogenization instead whole cells	Sasaki et al. (1998)
DNA strand breaks (alkaline comet assay)	Mouse, CD-1 (M)	Colon	±	*ortho*-Anisidine, 690 mg/kg bw	Oral, 1×	Using modified comet assay; nuclei isolated by homogenization instead of whole cells; results positive 3 h, but not 24 h, after *ortho*-anisidine administration	Sasaki et al. (1998)

Table 4.1 (continued)

End-point (assay)	Species, strain (sex)	Tissue	Results[a]	Agent, dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Gene mutation (*lacI*⁻ transgenic model)	Mouse, Big Blue™ (M)	Bladder	±	*ortho*-Anisidine hydrochloride, 750 mg/kg bw	Oral, 1×/day for 3 days	Statistical significance only after 14 days of administration using one-sided Student's *t*-test. Results were not confirmed in a repeat of the experiment	Ashby et al. (1994)
Gene mutation (*lacI*⁻ transgenic model)	Mouse, Big Blue™, (M)	Liver	–	*ortho*-Anisidine hydrochloride, 750 mg/kg bw	Oral, 1×/day for 10 days		Ashby et al. (1994)
Micronucleus formation	Rat, AP (M) and F344 (M)	Liver	–	*ortho*-Anisidine, 1104 mg/kg bw (AP); and 690 mg/kg bw (F344)	Oral, 1×		Ashby et al. (1991)
Micronucleus formation	Rat, AP (M)	Bone marrow	–	*ortho*-Anisidine, 1380 mg/kg bw	Oral, 1×		Ashby et al. (1991)
Micronucleus formation	Mouse, BDF_1 (M)	Bone marrow	±	*ortho*-Anisidine, 800 mg/kg bw	Intraperitoneal, 1×/day	The administered dose was lethal to CD-1 mice	Morita et al. (1997)
Micronucleus formation	Mouse, $B6C3F_1$ (M)	Bone marrow	–	*ortho*-Anisidine, 500 mg/kg bw	Intraperitoneal, 1×/day for 3 days		Ashby et al. (1991)
Micronucleus formation	Mouse, CBA (M)	Bone marrow	–	*ortho*-Anisidine, 690 mg/kg bw	Oral, 1×/day for 3 days		Ashby et al. (1991)
DNA repair (host-mediated assay)	Mouse, NMRI (M)	*Escherichia coli* K-12 in blood	–	*ortho*-Anisidine, 1300 mg/kg bw	Oral, 1×/day		Hellmér & Bolcsfoldi (1992b)
DNA repair (host-mediated assay)	Mouse, NMRI (M)	*Escherichia coli* K-12 in blood and kidney	+	*ortho*-Anisidine, 310 mg/kg bw	Intraperitoneal, 1×/day		Hellmér & Bolcsfoldi (1992b)

bw, body weight; d, day; F, female; HID, highest ineffective dose; HLB, hydrophilic–lipophilic-balanced; LED, lowest effective dose; M, male; wk, week.

[a] +, positive; –, negative; ±, equivocal (variable response in several experiments within an adequate study).

Rat and rabbit hepatic microsomal CYP enzymes catalyse both O-demethylation and N-hydroxylation of *ortho*-anisidine to form a reactive metabolite, *N*-(2-methoxyphenyl) hydroxylamine (Naiman et al., 2008a, b). As shown in Fig. 4.1, studies using human, rabbit, or rat hepatic microsomes reported CYP-dependent oxidation of *ortho*-anisidine to its major metabolite, *N*-(2-methoxyphenyl) hydroxylamine. This *N*-hydroxy compound can be further oxidized to *ortho*-nitrosoanisole or reduced back to *ortho*-anisidine. Moreover, studies in vitro using mammalian peroxidases showed CYP-dependent formation of the electrophilic species diimine and quinone imine. [The Working Group noted that the bioactivation of *ortho*-anisidine to electrophilic species involves N-oxidation by CYP-associated enzymes, and parallels an established paradigm for aromatic amines such as 4-aminobiphenyl, 2-naphthylamine, and *ortho*-toluidine, which have been classified as *carcinogenic to humans* (IARC Group 1) (IARC, 2010, 2012).]

4.2.2 Is genotoxic

Table 4.1, Table 4.2, and Table 4.3 summarize the available studies on the genetic and related effects of *ortho*-anisidine and *ortho*-anisidine hydrochloride.

(a) Humans

(i) Exposed humans

No data were available to the Working Group.

(ii) Human cells in vitro

DNA damage, analysed by the quantification of phosphorylated histone H2AX (γ-H2AX) in protein extracts of cells and by biased sinusoidal field-gel electrophoresis assay, was detected in 1T1 cells (human ureter epithelial cells immortalized by transfection with the human papillomavirus *E6* and *E7* genes) treated with *ortho*-anisidine (10 mM) for 4 hours. The generation of γ-H2AX increased in a dose-dependent manner after treatment of 1T1 cells with *ortho*-anisidine (5 mM) for 4 hours (Qi et al., 2020). Induction of γ-H2AX was also reported in human liver carcinoma HepG2 cells treated with *ortho*-anisidine (5 mM) for 4 hours. [The Working Group noted that the authors did not present quantification values for induction of γ-H2AX in HepG2 cells. HepG2 cells have low metabolic competence.]

(b) Experimental systems

(i) Non-human mammals in vivo

See Table 4.1.

The effect of *ortho*-anisidine on DNA damage was evaluated in several studies in rodents, and positive results in several tissues, including urinary bladder, were observed. Iatropoulos et al. (2015) observed a statistically significant increase in the mean percentage values of tail DNA, assessed by comet assay, in the urinary bladder of Wistar rats treated with *ortho*-anisidine hydrochloride for 1 month. Ashby et al. (1991) did not observe DNA strand breaks in the liver, thymus, testes, kidney, spleen, or urinary bladder of Wistar or Sprague-Dawley rats, after a single oral or intraperitoneal dose of *ortho*-anisidine. On the other hand, in Wistar rats, *ortho*-anisidine induced DNA strand breaks in the colon, kidney, bladder, and lung at the 3-hour sampling time; in the stomach, colon, kidney, bladder, lung, and brain at the 8-hour sampling time; and in the colon at the 24-hour sampling time after a single oral dose (Sekihashi et al., 2002). Wada et al. (2012) did not observe an increase in the frequency of DNA strand breaks in the urinary bladder of male and female Sprague-Dawley rats given a single dose of *ortho*-anisidine. Data from an international validation study on the comet assay technique indicated that a single dose of *ortho*-anisidine administered to Crl:CD(SD) male rats induced DNA strand breaks in the liver when the median percentage tail DNA, instead of the mean percentage tail DNA, was analysed

Table 4.2 Genetic and related effects of *ortho*-anisidine in non-human mammalian cells in vitro

End-point	Species, tissue, cell line	Results[a]		Concentration (LEC or HIC)	Reference
		Without metabolic activation	With metabolic activation		
DNA strand breaks (alkaline unwinding test)	Mouse lymphoma L5178Y cells	–	+	150 µg/mL	Garberg et al. (1988)
Gene mutation, *Tk*$^{+/-}$ locus	Mouse lymphoma L5178Y cells	+	+	123 µg/mL	Wangenheim & Bolcsfoldi (1988)
Chromosomal aberrations	Chinese hamster ovary cells	+	+	1200 µg/mL	Galloway et al. (1987)
Chromosomal aberrations	Chinese hamster lung	+	+	1000 µg/mL	JETOC (1997)
Sister-chromatid exchange	Chinese hamster ovary cells	+	+	38 µg/mL	Galloway et al. (1987)

HIC, highest ineffective concentration; LEC, lowest effective concentration; Tk, thymidine kinase.

[a] +, positive; –, negative.

Table 4.3 Genetic and related effects of *ortho*-anisidine and *ortho*-anisidine hydrochloride in non-mammalian and acellular experimental systems

Test system (species, strain)	End-point	Results[a]		Agent, concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium TA100	Reverse mutation	–	+	*ortho*-Anisidine, 10 µg/plate	No information on source of *ortho*-anisidine, no details on methodology	Shimizu & Takemura (1983)
Salmonella typhimurium TA98	Reverse mutation	–	+	*ortho*-Anisidine hydrochloride, 10 000 µg/plate	4 positive findings with RLI, MNL, MLI, and HLI	Dunkel et al. (1985)
Salmonella typhimurium TA100	Reverse mutation	–	+	*ortho*-Anisidine hydrochloride, 10 000 µg/plate	1 positive finding with MLI	Dunkel et al. (1985)
Salmonella typhimurium TA1535	Reverse mutation	–	–	*ortho*-Anisidine hydrochloride, 10 000 µg/plate		Dunkel et al. (1985)
Salmonella typhimurium TA1537	Reverse mutation	+	–	*ortho*-Anisidine hydrochloride, 10 000 µg/plate	1 positive finding with MLN	Dunkel et al. (1985)
Salmonella typhimurium TA1538	Reverse mutation	–	+	*ortho*-Anisidine hydrochloride, 10 000 µg/plate	6 positive findings with MLI and HLI	Dunkel et al. (1985)
Salmonella typhimurium WP2*uvrA*	WP2*uvrA*	–	–	*ortho*-Anisidine hydrochloride, 10 000 µg/plate		Dunkel et al. (1985)
Escherichia coli WP2*uvrA*	Reverse mutation	–	–	*ortho*-Anisidine, 10 000 µg/plate		Dunkel et al. (1985)
Salmonella typhimurium TA98, TA100	Reverse mutation	–	+	*ortho*-Anisidine, 100 µg/plate for TA98 and 33 µg/plate for TA100		Zeiger et al. (1992)
Salmonella typhimurium YG1012 (TA1538 with *N*-acetyltransferase gene)	Reverse mutation	–	–	*ortho*-Anisidine, NR		Thompson et al. (1992)
Salmonella typhimurium YG1029 (TA100 with *N*-acetyltransferase gene)	Reverse mutation	–	+	*ortho*-Anisidine, 62 µg/plate		Thompson et al. (1992)
Salmonella typhimurium TA1538	Reverse mutation	–	–	*ortho*-Anisidine, 100 µg/plate		Ferretti et al. (1977)
Salmonella typhimurium TA1538	Reverse mutation	–	–	*ortho*-Anisidine, 100 µg/plate		Garner & Nutman (1977)

Table 4.3 (continued)

Test system (species, strain)	End-point	Results[a]		Agent, concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium TA98, TA100, TA1535, TA1537	Reverse mutation	–	–	*ortho*-Anisidine, 10 800 µg/plate		Haworth et al. (1983)
Salmonella typhimurium TA98, TA100, TA1535, TA1537, TA1538, G46, C3076, D3052,	Reverse mutation	–	–	*ortho*-Anisidine, 10 000 µg/plate	No clear statement about dose range	Thompson et al. (1983)
Escherichia coli WP2, WP2*uvrA*	Reverse mutation	–	–	*ortho*-Anisidine, 10 000 µg/plate	No clear statement about dose range	Thompson et al. (1983)
Salmonella typhimurium TA102, TA2638	Reverse mutation	–	–	*ortho*-Anisidine, 5000 µg/plate		Watanabe et al. (1996)
Salmonella typhimurium NM2009 (with *O*-acetyltransferase gene)	SOS/*umuC* gene expression (DNA damage)	NR	–	*ortho*-Anisidine, 1000 µg/mL		Oda et al. (1995)
Salmonella typhimurium NM6001 (with *N*-acetyltransferase 1 gene or with *N*-acetyltransferase 2 gene)	SOS/*umuC* gene expression (DNA damage)	NR	+	*ortho*-Anisidine, 100 µM	Dosing not clearly reported	Oda (2004)
Escherichia coli K-12 343	DNA repair	+	–	*ortho*-Anisidine, 94.9 mM [11 687 µg/mL]		Hellmér & Bolcsfoldi (1992a)
Drosophila melanogaster	Sex-linked recessive lethal mutation assay	NA	– –	*ortho*-Anisidine, 500 ppm [500 µg/g], feeding *ortho*-Anisidine, 2000 ppm [2000 µg/mL], injection		Yoon et al. (1985)
Drosophila melanogaster	Interchromosomal mitotic recombination (somatic w/w+ eye assay)	+	NA	*ortho*-Anisidine hydrochloride, 0.5 mM [61.5 µg/mL]		Rodriguez-Arnaiz & Aranda (1994)
Drosophila melanogaster	Interchromosomal mitotic recombination (somatic w/w+ eye assay)	+	NA	*ortho*-Anisidine, 1 mM [123 µg/mL]	Negative results in insecticide-resistant strain	Rodriguez-Arnaiz & Téllez (2002)

Table 4.3 (continued)

Test system (species, strain)	End-point	Results[a]		Agent, concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Saccharomyces cerevisiae strain RS112	DNA deletion by intrachromosomal recombination (DEL assay)	+	NT	*ortho*-Anisidine, 5 mg/mL		Brennan & Schiestl (1999)
Acellular system, [^{14}C]-labelled *ortho*-anisidine	Calf thymus DNA covalent binding	NA	+	*ortho*-Anisidine, 1 mM		Thompson & Eling (1991)
Acellular system, [^{14}C]-labelled *ortho*-anisidine	Calf thymus DNA covalent binding	NA	+	*ortho*-Anisidine, 1 mM [123 µg/mL]		Stiborová et al. (2002)
Acellular system, ^{32}P-postlabelling	Calf thymus DNA covalent binding	NA	+	*ortho*-Anisidine, 1 mM [123 µg/mL]	Positive when 3× higher amounts of MN/SPD were used	Stiborová et al. (2002)

HIC, highest ineffective concentration; HLI, hamster liver induced S9; LEC, lowest effective concentration; MLI, mouse liver induced S9; MLN, mouse liver S9, not induced; MN, micrococcal nuclease; NA, not applicable; NR, not reported; NT, not tested; RLI, rat liver induced S9; S9, 9000 × *g* supernatant; SPD, spleen phosphodiesterase.

[a] +, positive; –, negative.

(Uno & Omori, 2015). [The Working Group noted that the comet assay resulted in equivocal responses in this study, i.e. positive depending on the choice of statistical evaluation method.] Hobbs et al. (2015) reported that *ortho*-anisidine induced DNA strand breaks in the liver, without a corresponding significant dose–response relationship, but did not induce DNA strand breaks in the stomach of Sprague-Dawley rats. Toyoda et al. (2019) reported that administration of *ortho*-anisidine hydrochloride to Fischer/DuCrl-Crlj rats for 1 month induced DNA damage, as analysed by the quantification of γ-H2AX-positive epithelial cells in the urinary bladder.

ortho-Anisidine or its hydrochloride form did not induce unscheduled DNA synthesis in Fischer 344 or AP rat liver or kidney (Tyson & Mirsalis, 1985; Ashby et al., 1991).

In ddY mice, *ortho*-anisidine induced DNA strand breaks in the colon and urinary bladder at the 3- and 8-hour sampling times and in the urinary bladder at the 24-hour sampling time (Sekihashi et al., 2002). In CD-1 mice, a single dose of *ortho*-anisidine induced DNA damage, assessed by a modified comet assay, in the urinary bladder and colon but not in the stomach, kidney, liver, lung, brain, or bone marrow (Sasaki et al., 1998).

ortho-Anisidine hydrochloride induced gene mutation in the *lacI* transgene; in the Big Blue™ mouse, there was a modest effect in the bladder and no effect in the liver (Ashby et al., 1994). *ortho*-Anisidine did not induce micronucleus formation in AP rat bone marrow or liver (Ashby et al., 1991) or in BDF1 or B6C3F$_1$ mouse bone marrow (Morita et al., 1997; Ashby et al., 1991).

ortho-Anisidine induced DNA repair in *Escherichia coli* in a host-mediated assay in male NMRI mice treated by intraperitoneal administration but not when treated by gavage (Hellmér & Bolcsfoldi, 1992b).

(ii) Non-human mammalian cells in vitro

See Table 4.2.

ortho-Anisidine did not induce unscheduled DNA synthesis in primary cultured rat hepatocytes (Thompson et al., 1983; Yoshimi et al., 1988).

Although DNA strand breaks were observed only in the presence of an exogenous metabolic system (Garberg et al., 1988), *ortho*-anisidine induced gene mutations in mouse lymphoma L5178Y cells in vitro both with and without exogenous metabolic activation (Wangenheim & Bolcsfoldi, 1988). Chromosomal aberrations and sister-chromatid exchange were induced in Chinese hamster ovary cells in vitro both with and without exogenous metabolic activation (Galloway et al., 1987). Structural chromosomal aberrations were observed in Chinese hamster lung cells both with and without exogenous metabolic activation (JETOC, 1997).

(iii) Non-mammalian experimental systems

See Table 4.3.

Three studies were conducted in *Drosophila melanogaster* (Yoon et al., 1985; Rodriguez-Arnaiz & Aranda, 1994; Rodriguez-Arnaiz & Téllez, 2002). *ortho*-Anisidine did not induce sex-linked recessive lethal mutations in *Drosophila* (Yoon et al., 1985), but this compound and its hydrochloride form induced, in a dose-dependent manner, the frequency of light spots in the eyes of an insecticide-sensitive *Drosophila* strain (white/white somatic assay), suggesting loss of heterozygosity by mitotic recombination (Rodriguez-Arnaiz & Aranda, 1994; Rodriguez-Arnaiz & Téllez, 2002).

ortho-Anisidine induced genotoxic effects by increasing recombination frequency, analysed by DEL recombination assay in *Saccharomyces cerevisiae* strain RS112 (Brennan & Schiestl, 1999).

ortho-Anisidine induced reverse mutations in *Salmonella typhimurium* strains TA98, TA100, TA1537, and TA1538, with exogenous metabolic activation (Shimizu & Takemura, 1983; Dunkel et al., 1985; Zeiger et al., 1992). In the presence of exogenous metabolic activation, *ortho*-anisidine

induced reverse mutations in strain YG1029 (but not in strain YG1012, both YG strains having elevated levels of *N*-acetyltransferase) (Thompson et al., 1992). *ortho*-Anisidine or its hydrochloride form did not induce reverse mutation in *E. coli* or in *S. typhimurium* strains TA98, TA100, TA102, TA1535, TA1537, TA1538, TA2638, G46, C3076, D3052, or YG1012 (Ferretti et al., 1977; Garner & Nutman, 1977; Haworth et al., 1983; Thompson et al., 1983, 1992; Dunkel et al., 1985; Zeiger et al., 1992; Watanabe et al., 1996). *ortho*-Anisidine induced dose-dependent expression of the *umuC* gene in *S. typhimurium* overexpressing *N*-acetyltransferase type 1 and *N*-acetyltransferase type 2 (Oda, 2004), but not in *S. typhimurium* overexpressing *O*-acetyltransferase (Oda et al., 1995).

ortho-Anisidine, without exogenous metabolic activation, preferentially killed DNA repair-deficient *E. coli* strains rather than repair-proficient strains (Hellmér & Bolcsfoldi, 1992a).

4.2.3 Alters cell proliferation, cell death, or nutrient supply

(a) Humans

No data were available to the Working Group.

(b) Experimental systems

Cell proliferation was not induced in the urinary bladder of Wistar rats treated with *ortho*-anisidine hydrochloride (17 mg/kg bw per day, 3 days per week, for 4 weeks) (Iatropoulos et al., 2015). Histopathological evaluation of the bladder of F344/DuCrl-Crlj rats treated with *ortho*-anisidine hydrochloride (1.0% in the feed, for 2 or 4 weeks) showed hyperplasia with an increase in the frequency of cells that tested positive for the cell proliferation marker, Ki67 (Toyoda et al., 2019).

Male and female Fischer 344 rats given feed containing *ortho*-anisidine hydrochloride at a concentration of 5000 or 10 000 mg/kg for up to 103 weeks developed non-neoplastic lesions of the thyroid gland and kidney more frequently than did control animals (NCI, 1978).

In female B6C3F$_1$ mice that received feed containing *ortho*-anisidine hydrochloride at a concentration of 2500 or 5000 mg/kg for up to 103 weeks, the incidence of cystic hyperplasia of the uterine endometrium was higher than in control mice. There was an increased incidence of hyperplasia of the bladder in male and female B6C3F$_1$ mice at 5000 mg/kg relative to controls (NCI, 1978).

4.2.4 Evidence relevant to other key characteristics of carcinogens

(a) Humans

In human liver carcinoma cell lines HepG2 and Huh-7 single cultured and co-cultured with human monocytic THP-1 cells, *ortho*-anisidine induced reactive oxygen species in a concentration-dependent manner, as measured by the 2′,7′-dichlorodihydrofluorescein diacetate assay (Wewering et al., 2017).

(b) Experimental systems

In the absence of exogenous metabolic activation, *ortho*-anisidine hydrochloride at the lowest effective dose (500 µg/mL) induced cell transformation in Syrian hamster embryo cells in vitro (Kerckaert et al., 1998).

Treatment of primary murine hepatocytes with *ortho*-anisidine at 2.5 or 10 mM led to a significant increase in levels of reactive oxygen species after 3 hours of incubation (Wewering et al., 2017). Brennan & Schiestl (1999) reported that the genotoxic effects of *ortho*-anisidine in yeast were reduced in the presence of the free radical scavenger and antioxidant *N*-acetyl cysteine. In addition, comparing yeast strains with different capacities for the detoxification of oxygen radicals, it was shown that a strain with an inactivating disruption in the superoxide dismutase genes *SOD1* and *SOD2* was hypersensitive

to the lethal effects of *ortho*-anisidine, which implies a role of the superoxide anion (O_2^-) in its cytotoxicity (Brennan & Schiestl, 1999). *ortho*-Anisidine also induced the production in yeast of reactive oxygen species, measured by the oxidation of the free radical-sensitive reporter compound 2′,7′-dichlorodihydrofluorescein diacetate (Brennan & Schiestl, 1999).

The *ortho*-anisidine metabolite, *ortho*-aminophenol, induced Cu(II)-dependent DNA damage. This result was achieved using ^{32}P-labelled human DNA fragments (of *c-Ha-RAS* and *TP53)* and calf thymus DNA. In the presence of Cu(II), *ortho*-aminophenol induced formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) in calf thymus DNA (Ohkuma & Kawanishi, 2001).

ortho-Anisidine (1232 µg/mL) inhibited gap-junctional intercellular communication in mouse keratinocytes in the absence of an exogenous metabolic system, which was related to the decreased intensity of immunocytochemical staining for protein connexin 43 on the cell membrane (Jansen et al., 1996), but did not inhibit gap-junctional intercellular communication in Syrian hamster embryo cells exposed to 0.03–10 mM *ortho*-anisidine for up to 24 hours (Rivedal et al., 2000).

4.3 Data relevant to comparisons across agents and end-points

The analysis of the in vitro bioactivity of the agents reviewed in the present volume was informed by data from high-throughput screening assays generated by the Toxicity Testing in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) research programmes of the government of the USA (Thomas et al., 2018). *ortho*-Anisidine was one of thousands of chemicals tested across the large assay battery of the Tox21 and ToxCast research programmes as of 26 April 2020. Detailed information about the chemicals tested, assays used, and associated procedures for data analysis is publicly available (US EPA, 2021). [The Working Group noted that the metabolic capacity of the cell-based assays is variable, and generally limited, as acknowledged in Kavlock et al. (2012).]

Among the 676 assays in which *ortho*-anisidine (at concentrations up to 100 µM) was tested, it was found to be inactive in almost all assays. Active responses were observed in 18 assays (US EPA, 2019b). Upregulation of the aryl hydrocarbon receptor (AHR) in the hepatocellular carcinoma-derived cell line, HepG2, was reported in two assays at the half-maximal activity concentration (AC_{50}) of 74.5 µM, and with borderline effects at an AC_{50} concentration of 63.5 µM. Inhibition of thyroid peroxidase was reported in two assays at AC_{50} concentrations of 0.318 and 27.8 µM in rat and pig thyroid gland cell lines, respectively. Activation of the pregnane X receptor, PXR (NR1I2), was reported in one assay at an AC_{50} concentration of 60.3 µM in HepG2 cells. Activation of the rat adrenoceptor alpha 2B (Adra2b) was reported in one assay at an AC_{50} concentration of 8.22 µM.

Borderline activity was reported for human retinoid X receptor beta (RXRB), protein tyrosine phosphatase non-receptor type 6 (PTPN6), protein tyrosine phosphatase receptor type C (PTPRC), protein phosphatase 1 catalytic subunit alpha isozyme (PPP1CA), mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2) and sirtuin 1 (SIRT1) at AC_{50} concentrations ranging from 0.164 to 41.3 µM. Mitochondrial depolarization was reported in one assay in HepG2 cells, at an AC_{50} concentration of 32.5 µM.

4.4 Other relevant evidence

ortho-Anisidine induced methaemoglobinaemia in CBA mice and Alpk:APfSD rats treated by oral administration (Ashby et al., 1991).

5. Summary of Data Reported

5.1 Exposure characterization

ortho-Anisidine, the parent compound of *ortho*-anisidine hydrochloride, is a basic compound and will undergo acid–base reactions. *ortho*-Anisidine and its hydrochloride salt will achieve a pH-dependent acid–base equilibrium in the body.

ortho-anisidine is a substituted aniline compound with the formula $NH_2C_6H_4OCH_3$. It is produced from *ortho*-nitroanisole (see the monograph on *ortho*-nitroanisole in the present volume). It is not a High Production Volume chemical, and its use has been declining in Europe and the USA. Little information is available about production or use in other regions. Its main use is as a chemical intermediate in the synthesis of azo pigments and dyes. These are then used in consumer products, textiles, and for printing paper and cardboard.

The salt, *ortho*-anisidine hydrochloride, is derived from *ortho*-anisidine and is not produced in significant quantities. It is also used as a chemical intermediate for production of dyes and pigments, and has some minor industrial uses.

Exposure to both *ortho*-anisidine and its hydrochloride form may occur through inhalation, ingestion, and skin and eye contact. Time-weighted average occupational exposure limits for *ortho*-anisidine have been established in a number of countries. Sparse measurements in production plants have shown exposure near the exposure limit values, with measured exposures in pigment plants being much lower.

ortho-Anisidine has been detected during product testing of textiles and consumer products and has also been found in tattoo inks and cigarette smoke, and in urine samples from the general population.

5.2 Cancer in humans

The research available related to cancers in humans was limited to one case series of bladder cancer occurring in plants producing organic dye and pigment intermediates. All 3 cases exposed to *ortho*-anisidine were co-exposed to other known bladder carcinogens (*ortho*-toluidine, tobacco smoking).

The available study did not permit a conclusion to be drawn about the presence of a causal association between *ortho*-anisidine and urinary bladder cancer.

5.3 Cancer in experimental animals

ortho-Anisidine hydrochloride caused an increase in the incidence of malignant neoplasms in two species.

In $B6C3F_1$ mice, *ortho*-anisidine hydrochloride administered orally (in feed) in one study caused an increase in the incidence of transitional cell carcinoma of the urinary bladder in males and females.

In Fischer 344 rats, *ortho*-anisidine hydrochloride administered orally (in feed) in one study caused an increase in the incidence of transitional cell carcinoma of the urinary bladder in males and females, and of transitional cell carcinoma of the renal pelvis in males. In addition, *ortho*-anisidine hydrochloride caused an increase in the incidence of a combination of benign and malignant neoplasms (follicular cell carcinoma, adenoma, cystadenoma, papillary cystadenoma, or papillary cystadenocarcinoma, combined) of the thyroid in male rats.

5.4 Mechanistic evidence

No studies characterizing the absorption, distribution, metabolism, or excretion of *ortho*-anisidine or *ortho*-anisidine hydrochloride in humans were available. In two studies of people without known exposure, *ortho*-anisidine

was detected in the urine. Studies using human, rabbit, or rat hepatic microsomes reported cytochrome P450-dependent oxidation of *ortho*-anisidine to its major metabolite, *N*-(2-methoxyphenyl)hydroxylamine. This *N*-hydroxy compound can be further oxidized to *ortho*-nitrosoanisole or reduced back to *ortho*-anisidine. *ortho*-Anisidine can also be metabolized to *ortho*-aminophenol via demethylation. Studies in vitro using mammalian peroxidases showed cytochrome P450-dependent formation of electrophilic species, diimine and quinone imine. In male IMP:WIST rats with intraperitoneal exposure, *ortho*-anisidine was readily absorbed, widely distributed to tissues, and excreted primarily via the urine as *N*-acetyl-2-methoxyaniline.

There is consistent and coherent evidence that *ortho*-anisidine or its hydrochloride form exhibit key characteristics of carcinogens in experimental systems. *ortho*-Anisidine is metabolically activated to electrophiles. Haemoglobin adducts have been detected in blood samples from exposed humans in two studies. No data on DNA adducts in exposed humans were available. In studies in vitro, *ortho*-anisidine is activated by human hepatic microsomes to form DNA adducts. The major adduct formed was *N*-(deoxyguanosin-8-yl)-2-methoxyaniline. In experimental systems, *ortho*-anisidine DNA adducts were observed in several tissues of rats, with the highest level found in the urinary bladder. After peroxidation to diimine and quinone imine, *ortho*-anisidine binds to calf thymus DNA, in the presence of mammalian microsomes. *ortho*-Anisidine is genotoxic, based on the results of multiple studies demonstrating the formation of DNA strand breaks in the bladder of rodents, and mutagenic in base-pair substitution strains of bacteria, both in the presence and in the absence of exogenous metabolic activation.

ortho-Anisidine hydrochloride alters cell proliferation, cell death, or nutrient supply. *ortho*-Anisidine hydrochloride induced hyperplasia in the bladder of rodents – in male F344/DuCrl-Crlj rats after short-term exposure, and in male and female B6C3F$_1$ mice after chronic exposure. It also induced cystic hyperplasia of the uterine endometrium of female B6C3F$_1$ mice.

ortho-Anisidine was mostly without effects in the assay battery of the Tox21 and ToxCast research programmes.

Overall, the evidence is consistent and coherent that *ortho*-anisidine belongs, on the basis of mechanistic considerations, to a class of aromatic amines. Members of this class, including 4-aminobiphenyl, 2-naphthylamine, and *ortho*-toluidine have been classified previously by the *IARC Monographs* programme as *carcinogenic to humans* (IARC Group 1). *ortho*-Anisidine is structurally similar to these aromatic amines. *ortho*-Anisidine is also similar to these aromatic amines with respect to its mechanism of bioactivation to electrophiles, genotoxicity, and target organs of carcinogenicity in chronic animal bioassays. The urinary bladder is a common target organ of carcinogenicity for these aromatic amines in experimental animals. For instance, *ortho*-anisidine causes malignant tumours of the urinary bladder when administered orally to rats, as do *ortho*-toluidine and 2-naphthylamine. 4-Aminobiphenyl causes malignant tumours of the urinary bladder when administered orally to dogs and mice. Therefore, these mechanistic considerations go beyond chemical structural similarity to encompass biological and biochemical similarities relevant to common key characteristics of carcinogens.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *inadequate evidence* in humans regarding the carcinogenicity of *ortho*-anisidine and *ortho*-anisidine hydrochloride.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of *ortho*-anisidine hydrochloride.

6.3 Mechanistic evidence

There is *strong evidence* that *ortho*-anisidine belongs, based on mechanistic considerations, to a class of aromatic amines for which several members have been classified as carcinogenic to humans. There is also *strong evidence* that *ortho*-anisidine exhibits key characteristics of carcinogens in experimental systems.

6.4 Overall evaluation

ortho-Anisidine and *ortho*-anisidine hydrochloride are *probably carcinogenic to humans (Group 2A)*.

6.5 Rationale

The Group 2A evaluation is based on *strong* mechanistic evidence that *ortho*-anisidine, on the basis of mechanistic considerations, belongs to a class of aromatic amines for which several members have been classified as carcinogenic to humans. *ortho*-Anisidine bears structural similarity to other members of this class, and there is close concordance with respect to the bioactivation mechanism to DNA-reactive moieties, genotoxicity, and target organs of carcinogenicity in chronic animal bioassays.

There is also *sufficient evidence of carcinogenicity* in experimental animals on the basis of increased incidence of malignant neoplasms in two species.

In addition, there is *strong evidence* that *ortho*-anisidine exhibits key characteristics of carcinogens in experimental systems. *ortho*-Anisidine is metabolically activated to electrophiles, it is genotoxic, and it alters cell proliferation, cell death, or nutrient supply.

The evidence for cancer in humans is *inadequate* as the only data available were from a single case series of bladder cancer in workers who were co-exposed to other bladder carcinogens (*ortho*-toluidine, tobacco smoking).

ortho-Anisidine hydrochloride is in equilibrium with *ortho*-anisidine; therefore, the classification of carcinogenic hazard applies to both *ortho*-anisidine and its hydrochloride form.

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