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2,4-Toluenediamine

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A. Hartwig[1,*] MAK Commission[2,*]

1 *Chair of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Institute of Applied Biosciences, Department of Food Chemistry and Toxicology, Karlsruhe Institute of Technology (KIT), Adenauerring 20a, Building 50.41, 76131 Karlsruhe, Germany*

2 *Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, Deutsche Forschungsgemeinschaft, Kennedyallee 40, 53175 Bonn, Germany*

* *email: A. Hartwig (andrea.hartwig@kit.edu), MAK Commission (arbeitsstoffkommission@dfg.de)*

Keywords

2,4-toluenediamine; genotoxicity; germ cell mutagenicity; clastogenicity; DNA adducts; liver

Abstract

The German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area has re-evaluated data for the genotoxicity of 2,4-toluenediamine [95-80-7]. 2,4-Toluenediamine is clastogenic in vitro. In vivo, it is clastogenic in the liver, the target organ of carcinogenicity, but not in the bone marrow or germ cells. DNA adducts were induced in vitro and in vivo. 2,4-Toluenediamine demonstrates mutagenic potential in vitro and in vivo. The latter is evident only in the liver, but not in the kidneys. There are no data for the mutagenic potential of the substance in germ cells. Although the germ cells may be reached, no evidence of the active form was found in the germ cells. Therefore, 2,4-toluenediamine has been classified in Germ Cell Mutagenicity Category 3B.

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MAK value	–
Peak limitation	–
Absorption through the skin (2005)	**H**
Sensitization (2004)	**Sh**
Carcinogenicity (1985)	**Category 2**
Prenatal toxicity	–
Germ cell mutagenicity (2020)	**Category 3 B**
BAR	not yet established

EKA (1999)

air 2,4-toluenediamine [mg/m³]	urine 2,4-toluenediamine (after hydrolysis) [µg/g creatinine]
0.0025	6
0.01	13
0.017	20
0.035	37
0.100	100

CAS number 95-80-7

For 2,4-toluenediamine, documentation (Henschler 1993) and supplements on sensitization (Hartwig 2014 a) and dermal absorption (Hartwig 2014 b) are available. In 1999, EKA were established (Lewalter 2010); a BAR, however, could not be derived (Nasterlack 2016). In this supplement, the germ cell mutagenicity of 2,4-toluenediamine is assessed. The reason is the evaluation of 2,4-toluene diisocyanate (Hartwig and MAK Commission 2023), from which 2,4-toluenediamine is formed as a hydrolysis product.

This supplement is based mainly on the Risk Assessment Report of the EU (2008). More recent studies that were not yet included and partly concern also other end points are presented in detail.

Toxicokinetics and Metabolism

2,4-Toluenediamine is hydroxylated mainly at the aromatic ring; 5-hydroxy-2,4-diaminotoluene is the main product of the aminophenols formed. In addition, *N*-acetylation or glucuronidation occurs (Henschler 1993). *N*-Oxidation to form hydroxylamine compounds takes place only to a small extent (Lewalter 2010).

The biotransformation of 2,4-toluenediamine in intact skin ex vivo, in various skin models and in cultured skin cells in vitro was compared. Reconstructed human full-thickness skin, human skin samples, primary epidermal keratinocytes and dermal fibroblasts, as well as epidermal Langerhans cells and dermal dendritic cells differentiated from monocytes in vitro were used. The only detectable metabolite was *N*-(3-amino-4-methylphenyl)acetamide formed by *N*-acetylation. The concentration of the metabolite was highest in reconstructed human whole skin, followed by human skin samples. In cell cultures, keratinocytes were the most active, followed by fibroblasts, Langerhans cells and dendritic cells, which produced similarly high concentrations of the metabolite (Grohmann et al. 2017).

Genotoxicity

The following is a summary of the results. The detailed presentation of the studies and their results can be found in EU (2008). More recent studies that were not yet included are described in detail below.

In vitro

2,4-Toluenediamine induced mutations in the Salmonella typhimurium strains TA98, TA100, TA1537 and TA1538 in the presence of a metabolic activation system at concentrations of 20 µg/plate and above. The available studies revealed strong concentration-dependent effects; toxic effects were not observed. Without a metabolic activation system, the results were negative up to 10 000 µg/plate. With the Salmonella strain TA1535 and Escherichia coli WP2uvrA, no mutations were induced up to concentrations of 10 000 and 5000 µg/plate, respectively, regardless of the presence or absence of the metabolic activation system. In the Salmonella strain TA97, 2,4-toluenediamine was likewise negative up to 500 µg/plate in the presence of a metabolic activation system. Bacterial acetyltransferase appears to play a critical role in the generation of the ultimate reactive product, as shown in a study with the Salmonella strain TA98 (wild type) and acetyltransferase-deficient (TA98/1,8-DNP6) or overexpressing strains (TA98(pYG219)). Compared with that induced in the wild-type strain, there was a 90% reduction in mutagenicity in the deficient strain, and a significant increase in mutagenicity in the overexpressing strain (EU 2008). A more recent study confirmed this observation using Salmonella typhimurium TA98 and YG1024, a strain overexpressing *O*-acetyltransferase, in the presence of a metabolic activation system (Toyoda-Hokaiwado et al. 2010).

2,4-Toluenediamine induced SCE (sister chromatid exchange) in CHO cells (a cell line derived from Chinese hamster ovary) and DNA strand breaks in human skin fibroblasts and V79 cells, but not in primary rat hepatocytes. In primary rat hepatocytes and single-stranded calf thymus DNA, also DNA adducts were formed after incubation with 2,4-toluenediamine. UDS (unscheduled DNA synthesis) assays with 2,4-toluenediamine in primary cultures of rat hepatocytes and human hepatocytes likewise yielded positive results, but data for cytotoxicity are not available. UDS was not induced in primary rat spermatocytes or spermatids after the *S* phase, but the assay was performed only without the addition of a metabolic activation system. Evidence of clastogenicity was found in the induction of chromosomal aberrations in CHO and CHL (a cell line derived from Chinese hamster lung) cells. Data for cytotoxicity are available for only one of four tests: in CHO cells, chromosomal aberrations were induced at concentrations of 2 mM (246 µg/ml) and above, and cytotoxicity occurred at 4 mM (490.8 µg/ml) and above (EU 2008).

As it is known to be a DNA-reactive genotoxic carcinogen, 2,4-toluenediamine is recommended as one of the model substances to test the sensitivity or specificity of new genotoxicity assays (Kirkland et al. 2016). Since the substance penetrates through the skin and is also metabolized in the skin, 2,4-toluenediamine was used to optimize the comet assay with the human epidermis model EpiDerm^{TM}. For this purpose, test series were performed in three different laboratories. The incubation period was 3 hours in each case. In all test laboratories, 2 of 3 experiments yielded positive results (Reus et al. 2013).

2,4-Toluenediamine induced mutations in mammalian cells only at cytotoxic concentrations. In studies with L5178Y mouse lymphoma cells and CHO-AT3-2 cells (*tk* and *hprt* locus) and V79 cells (*hprt* locus), a small increase in mutations occurred only in the TK$^{+/-}$ assay with the mouse lymphoma cells at highly cytotoxic concentrations and only without the addition of a metabolic activation system. The concurrent positive controls demonstrated the sensitivity of the test systems (EU 2008).

In vivo

Drosophila melanogaster

Sex-linked recessive lethal mutations were induced in Drosophila melanogaster adult males after dietary administration of 2,4-toluenediamine (concentrations up to 1857 µg/ml) for 3 days or after single doses by microinjection (concentrations up to 2443 µg/ml) (EU 2008).

Mammals

Indicator tests

A slight, not dose-dependent increase in the frequency of sister chromatid exchange was observed in the bone marrow of mice given single intraperitoneal doses of 9 and 18 mg/kg body weight. Higher doses resulted in severe local toxicity (no other details) (EU 2008).

In mice given single oral doses of 60 mg/kg body weight, DNA strand breaks were observed in the liver, kidneys and stomach, but not in the lungs, bone marrow, colon, bladder and brain. After intraperitoneal injection of 240 mg/kg body weight, DNA strand breaks were detected in the liver, kidneys and lungs, but not in the bone marrow and spleen. In rats, DNA strand breaks were induced in the stomach, colon, kidneys and brain after oral doses of 130 mg/kg body weight (50% of the LD_{50}). Negative results were obtained for the liver, bladder, lungs and bone marrow. The strand breaks were investigated using the comet assay. Another method, which made use of the different viscosity of DNA or DNA fragments, led to the detection of DNA strand breaks in the hepatocytes of rats treated once intraperitoneally. Doses up to the LD_{50} (147 mg/kg body weight) were used. Data for toxicity were not reported in these studies (EU 2008).

Groups of 5 male Sprague Dawley rats were given gavage doses of 2,4-toluenediamine of 0, 37.5, 75 or 150 mg/kg body weight and day at intervals of 24 and 21 hours on 3 consecutive days. In the stomach and liver, the induction of strand breaks was investigated by comet assay. To clarify the results obtained, a second study was performed with 0, 100, 150 or 200 mg/kg body weight and day. No DNA damage was induced in the stomach. In the liver, there was a small, statistically significant, increase in DNA strand breaks in the first study after the evaluation of 150 cells per animal in the high dose group. In the second study, the percentage of DNA in the tail was increased in a statistically significant, dose-dependent manner starting at the lowest dose tested of 100 mg/kg body weight and day. There was no increase in hedgehog cells, an indicator of cytotoxicity or severe genotoxic injury, in either liver or stomach. The animals displayed hepatocellular hypertrophy and hypertrophy or hyperplasia of the bile ducts and necrosis at 75 mg/kg body weight and day and above. No histopathological damage was observed in the stomach (De Boeck et al. 2015).

DNA adducts were detected in various organs of rats given 2,4-toluenediamine as single intraperitoneal injections or in the diet for up to 6 weeks. DNA adducts were detected mainly by ^{32}P postlabelling. In the feeding study, the maximum adduct formation in the liver and mammary glands was reached at the highest dose tested of 22.1 mg/kg body weight and day after 6 weeks; the maximum relative adduct level was about 200 per 10^7 nucleotides. In the studies with intraperitoneal injections of up to 250 mg/kg body weight, DNA adducts were found in the kidneys and lungs to a much lesser extent than in the liver and mammary gland, both target organs of carcinogenicity. All four organs had one main adduct and two other adducts at much lower levels. Adduct formation was highest in the liver and was dose-dependent. No DNA adducts were detected in T lymphocytes from the spleen of male rats after 32 weeks of dietary administration. No DNA adducts were detected with ^{3}H-labelled 2,4-toluenediamine in the liver of intraperitoneally treated rats. No information on toxicity is provided in any of the studies (EU 2008).

In rat liver, the single oral dose of 150 mg/kg body weight led to one weakly positive and one clearly positive result in the UDS test. A test with 300 mg/kg body weight yielded negative results. Data for liver toxicity are not provided in any of the studies (EU 2008).

Micronucleus tests

After a single intraperitoneal injection, no micronuclei were induced in the peripheral blood of male BDF1 mice up to a dose of 240 mg/kg body weight. Male PVG rats were given single oral 2,4-toluenediamine doses of 0, 150, 225 or 300 mg/kg body weight. Only after treatment with the high dose equivalent to the LD_{50} was there a doubling of the incidence of micronuclei in polychromatic erythrocytes after 24 hours, but not after 48 hours. Since half of the animals had died by this time point in the study, the result is of only minor relevance for the evaluation of genotoxic effects. In a second negative study carried out by the same research group in male and female F344 rats with single oral doses of 2,4-toluenediamine, cytotoxicity in the bone marrow was observed at the highest dose tested of 150 mg/kg body weight which manifested itself in a decreased ratio of polychromatic erythrocytes to normochromatic erythrocytes. The animals were treated with doses between 50 and 150 mg/kg body weight, controls received the solvent water (EU 2008; George and Westmoreland 1991).

The induction of micronuclei was likewise not observed in the peripheral blood reticulocytes of male F344-gpt delta transgenic rats fed diets containing 2,4-toluenediamine for 90 days. The concentrations in the diet were 0, 125, 250 or 500 mg/kg diet, with the high dose group receiving only 400 mg/kg diet from week 9 onwards due to reduced body weights (0, about 11.25, 22.5 or 41.5 mg/kg body weight, conversion factor 0.09 (for subchronic exposure) according to EFSA (2012)) (Toyoda-Hokaiwado et al. 2010).

In the liver of male Crl:CD (SD) rats, there was a statistically significant increase in micronucleated hepatocytes after oral doses of 0, 25 or 50 mg/kg body weight for up to 28 days. Each group consisted of 5 adult animals, and 2000 parenchymal hepatocytes were counted per animal. The low dose group was evaluated after 28 days of treatment, and the animals in the high dose group were evaluated after 5, 14 and 28 days; 2 animals died in this dose group (after 13 and 24 days). In the group given 25 mg/kg, the body weight was reduced to 95% of the value for the control group and in the group given 50 mg/kg it was reduced to 90%. A statistically significant increase in the incidence of micronuclei was found after 14 days of treatment (Narumi et al. 2012). The liver is one of the target organs of the carcinogenicity of 2,4-toluenediamine.

Dominant lethal tests

Groups of 20 male DBA/2J mice were given 2,4-toluenediamine doses of 0 or 40 mg/kg body weight orally or by intraperitoneal injection twice at 24-hour intervals. The mating period with 3 untreated females per week lasted 7 weeks in total. No dominant lethal mutations were observed after either oral or intraperitoneal administration. No morphological abnormalities were found in sperm examined 8 weeks after the treatment (EU 2008).

Mutagenicity tests

Ten-day treatment of male and female BigBlue^{TM} mice (C57BL/6) with gavage doses of 2,4-toluenediamine of 80 mg/kg body weight and day resulted in a doubling of the spontaneous mutation frequency in the liver *lacI* gene 28 days after the last administration (male animals: 8.46×10^{-5}, controls: 4.32×10^{-5}; female animals: 9.67×10^{-5}, controls: 4.32×10^{-5}). Ten days after the end of treatment, a slight increase in the mutation frequency was observed only in females (7.48×10^{-5}, control: 5.15×10^{-5}) and cell proliferation was increased in both sexes. The dose used was the maximum tolerable dose (MTD) according to a preliminary study in non-transgenic mice (EU 2008).

Dietary administration of 123 mg/kg body weight and day for 30 days did not significantly increase the mutation frequency in the *lacI* gene of liver DNA in male BigBlue^{TM} mice (B6C3F1), whereas 90-day treatment resulted in an increase from 5.7×10^{-5} in the controls to 12.1×10^{-5}. The dose used corresponded to the highest non-toxic dose in a 90-day study (EU 2008).

Dermal application of the MTD of 200 mg/kg body weight and day for 28 days, followed by a 7-day fixation period, induced mutations in the liver but not in the skin of male transgenic mice (Muta^{TM} mouse, CD2-*lacZ*80/HazfBR). The frequency of mutations in the kidney was marginally increased. One of 6 treated mice died and 3 exhibited decreased

body weights throughout the course of the study. The MTD was determined in a preliminary trial with dermal application for 3 to 7 days (Kirkland and Beevers 2006).

In the previously described 90-day feeding study in male transgenic rats, 2,4-toluenediamine (purity 95%) induced gene mutations at the *gpt* locus in the liver but not in the kidneys. The concentrations used were 0, 125, 250 or 500 mg/kg diet, with the high group receiving only 400 mg/kg diet from week 9 due to reduced body weights (0, about 11.25, 22.5 or 41.5 mg/kg body weight, conversion factor 0.09 (for subchronic exposure) according to EFSA (2012)). The predominant base substitutions were G:C to A:T transitions and G:C to T:A and G:C to C:G transversions. In addition, also base substitutions at A:T base pairs were induced. Furthermore, Spi^- deletion mutations were induced in the liver at 22.5 and 41.5 mg/kg body weight. Sequence analysis of plaques in the high dose group showed that most of them were frameshift mutations by one base, whereas the number of large deletion mutations was not increased. Hypertrophy and vacuolar degeneration were observed in hepatocytes even at the lowest dose tested of 11.25 mg/kg body weight and day, but increased cell proliferation was induced only at 22.5 mg/kg body weight and day. The 2,6-toluenediamine tested in parallel did not induce histopathological changes or mutations in the liver or kidneys (Toyoda-Hokaiwado et al. 2010).

Accessibility of the germ cells

The results of dominant lethal tests with oral or intraperitoneal administration were negative. No morphological abnormalities were found in the spermatozoa of the mice examined 8 weeks after treatment.

Intraperitoneal injection of 111 to 375 mg/kg body weight in male C57BL/6×C3H (B6C3F1) mice resulted in a dose-dependent decrease in DNA synthesis in the testes with a concomitant dose-dependent decrease in body temperature (EU 2008). Because of this parallel course, the decrease in DNA synthesis cannot be considered as evidence of DNA reactivity in the testes.

Manifesto (MAK value/classification)

Germ cell mutagenicity. 2,4-Toluenediamine is clastogenic in vitro, and indicator tests provided evidence of clastogenicity in vivo, which was confirmed only in the target organ the liver by the induction of micronuclei. In the bone marrow and in rodent germ cells, clastogenicity tests yielded negative results. DNA adducts are formed both in vitro and in vivo. 2,4-Toluenediamine has mutagenic potential in vitro and in vivo, the latter only in the liver and combined with the delayed induction of cell proliferation. The decrease in DNA synthesis in the testes of mice in the presence of hypothermia is not evidence of DNA reactivity, especially since other germ cell tests such as the two dominant lethal tests yielded negative results. Since the end point mutagenicity in germ cells has not been tested and the accessibility of the germ cells seems possible, but there is no evidence of the active form in the germ cells, 2,4-toluenediamine has been classified in Category 3B for germ cell mutagens.

Notes

Competing interests

The established rules and measures of the Commission to avoid conflicts of interest (www.dfg.de/mak/conflicts_interest) ensure that the content and conclusions of the publication are strictly science-based.

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