



Opinion of the Panel on Food Additives, Flavourings, Processing Aids, Materials in Contact with Food and Cosmetics of the Norwegian Scientific Committee for Food Safety

Adopted 11 January 2006

Risk assessment of health hazards from 4,4'-methylene dianiline (4,4'-MDA) migrated from polyamide cooking utensils

SUMMARY

The Norwegian Food Safety Authority [*Mattilsynet*] asked The Norwegian Scientific Committee for Food Safety [*Vitenskapskomiteen for mattrygghet* (VKM)] to issue an opinion on the risk linked to intake of 4,4'-methylene dianiline (4,4'-MDA, CAS no. 101-77-9 for the free base, CAS no. 13552-44-8 for the dihydrochloride salt), based on values of 4,4'-MDA found to migrate to water simulants from certain polyamide/nylon cooking utensils collected from the Norwegian market in a survey conducted by the Norwegian Food Safety Authority in 2004. The case was evaluated by the Panel on Food Additives, Flavourings, Processing Aids, Materials in Contact with Food and Cosmetics.

In the survey, three different cooking utensils from two separate kits, a ladle, a pasta spoon and a balloon whisk, were found to exceed the limit for migration of primary aromatic amines, expressed as aniline (not detectable, i.e. 0.02 mg/kg food or food simulant, according to the EU plastics directive 2002/72/EC), with levels of 42, 93 and 1089 $\mu\text{g}/\text{dm}^2$, respectively. 4,4'-MDA is listed in the Synoptic document on SCF list 4A (substances for which an ADI or TDI could not be established, but which could be used if the substance migrating into food simulants is not detectable by an agreed sensitive method). 4,4'-MDA is carcinogenic in rats and mice, and is classified by IARC in group 2B - possibly carcinogenic to humans, and it is found to be genotoxic *in vitro* and *in vivo*.

Based on the highest level of 4,4'-MDA found to migrate from a cooking utensil (1089 $\mu\text{g}/\text{dm}^2$) and a worst case intake scenario, a human intake of 15.6 $\mu\text{g}/\text{kg}$ bw/day was estimated. 4,4'-MDA is genotoxic and carcinogenic in experimental animals, and is to be considered a non-threshold carcinogen. By using the incidence of neoplastic nodules in the liver of male rats given 4,4'-MDA in drinking water for two years, the dose descriptors BMDL10 and T25 were calculated and used in the risk characterization. When the human cancer risk was evaluated by a margin of exposure (MOE) approach using the dose descriptor BMDL10, the MOE was only 109. Alternatively, a lifetime cancer risk for humans of 2.3×10^{-3} was quantitated using the dose descriptor T25. Based on both of these values, we consider the level of migration of 4,4'-MDA from these kitchen utensils to be of major concern. The same conclusion was reached by using an alternative exposure estimated by Danish authorities, or levels of migration detected elsewhere in Europe.

BACKGROUND

This evaluation was conducted in the context of the amounts of 4,4'-MDA found to migrate to water simulants from some of the cooking utensils collected from the Norwegian market in a survey conducted by the Norwegian Food Safety Authority in 2004 (1). Three different cooking utensils from two separate kits, a ladle, a pasta spoon and a balloon whisk, were found to exceed the limit for migration of primary aromatic amines, expressed as aniline (not detectable, i.e. 0.02 mg/kg food or food simulant, according to the Commission Directive 2002/72/EC (2)), with levels of 42, 93 and 1089 $\mu\text{g}/\text{dm}^2$, respectively. 4,4'-MDA is listed in the Synoptic document on SCF list 4A (substances for which an ADI or TDI could not be established, but which could be used if the substance migrating into food simulants is not detectable by an agreed sensitive method) (3). The origin of 4,4'-MDA in these cooking utensils is unknown, but may possibly be linked to colourants, such as aniline, or to its use as a chemical intermediate or hardener/curing agent in manufacture of the polyamide/nylon (4).

There have been several instances of primary aromatic amine related RASFF alerts notified by various countries in the EU in 2005 regarding cooking utensils, often produced in China. It is speculated whether the 4,4'-MDA stems from nylon not intended for food contact which has been reused in production of these cooking utensils (5). The levels of 4,4'-MDA, based on a selection of these alerts reporting 12 determined values, varied from 21 - 2067 $\mu\text{g}/\text{dm}^2$, i.e. from approximately half of the lowest value to twice the highest value found in the Norwegian study.

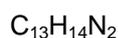
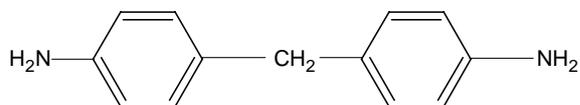


Figure 1. Structure of 4,4'-methylene dianiline (4,4'-MDA).

TERMS OF REFERENCE

In its letter of 23 February 2005 the Norwegian Food Safety Authority asked the Norwegian Scientific Committee for Food Safety to issue an opinion on the risk linked to intake of 4,4'-methylene dianiline (4,4'-MDA, CAS no. 101-77-9 for the free base, CAS no. 13552-44-8 for the dihydrochloride salt), based on values of 4,4'-MDA found to migrate to water simulants from some of the cooking utensils collected from the Norwegian market in a survey conducted by the Norwegian Food Safety Authority in 2004 (1). The case was evaluated by the Panel on Food Additives, Flavourings, Processing Aids, Materials in Contact with Food and Cosmetics.

RISK ASSESSMENT

Hazard characterization

Those studies for which the original paper could not be obtained, or that were not written in English, are referred in the following as stated in references 4, 6, 7 or 8.

Pharmacokinetics

Humans

Among 27 workers producing 4,4'-MDA, the percentage of urine samples found to contain 4,4'-MDA was 14.9% (levels > 200 µg/l) in 1970 and 0.09% (levels > 20 µg/l) in 1980 (9).

Human hepatic *N*-acetyl transferase can acetylate 4,4'-MDA, and *N*-acetyl-MDA was a major metabolite of 4,4'-MDA in the urine of 20 exposed workers (10). It appears to be excreted, at least in part, as a heat-labile conjugate, possibly a *N*-glucuronide. Urinary concentrations of monoacetyl-MDA were between 20 and 160 % of that of 4,4'-MDA, and it was shown that acetylation reduced the mutagenicity of 4,4'-MDA in *Salmonella typhimurium* TA100 after metabolic activation (11). This finding may have implications for humans with the slow acetylator phenotype.

4,4'-MDA and the stable metabolites *N*-acetyl-MDA (MAMDA) and *N,N'*-diacetyl-MDA (DAMDA) were determined in non-hydrolyzed post-shift urine from 63 workers exposed to 4,4'-MDA, and total 4,4'-MDA in urine after alkaline hydrolysis (12). Their relative concentrations (arithmetic means) were in the following order: total MDA > MAMDA > MDA > DAMDA. MAMDA represented more than 50% of total MDA, whereas MDA and DAMDA were lower than 15 and 3%, respectively. Acetylation of 4,4'-MDA, possibly a detoxification pathway, is confirmed to be an important metabolization route in humans, essentially through the monoacetylated metabolite. However, the individual ratio of MAMDA/total MDA, which can be related to the acetylator phenotype, was found to vary widely (roughly from 0 to 100 %).

Determination of hemoglobin adducts of 4,4'-MDA has been used for biomonitoring in humans, reflecting the internal dose absorbed by all routes after cumulative exposures (13). Hemoglobin adducts of 4,4'-MDA were detected in 31 of 33 exposed workers, and both MDA and *N*-acetyl-MDA were found in the urine of 20 of these individuals by GC-MS. Urinary levels of MDA, but not *N*-acetyl-MDA, correlated well with the hemoglobin adducts.

Animals

A single i.p. dose of 2, 10, 50 and 100 mg/kg bw 4,4'-MDA (99% pure) caused a dose-dependent increase in ethoxyresorufin-*O*-deethylation, ethoxycoumarin-*O*-deethylation and epoxide hydrolyation, and a concomitant decrease in aldrin epoxidase activity, in the liver of male Sprague-Dawley rats (14).

In an *in vivo* study with oral application of 4,4'-MDA to male Sprague-Dawley rats (50 mg/kg bw), *N*-acetyl-MDA was shown to be the major metabolite in urine (15). Minor amounts of *N,N'*-diacetyl-MDA and free MDA were also detected. Excretion of the *N*-acetyl- and *N,N'*-diacetyl-MDA in urine was mostly completed during 72 hours after administration.

Female Wistar rats were injected i.p. with 4,4'-MDA in dose ranges of 1-100 mg/kg bw or with ^{14}C -4,4'-MDA at 1 mg/kg bw to study the binding of 4,4'-MDA to hemoglobin (16). The sulphinic acid amide was considered to be one of the major hemoglobin adducts after reaction of 4,4'-MDA with cysteine residues in hemoglobin. A linear relationship was established between 4,4'-MDA dose and the amount of adduct cleaved from hemoglobin.

Female Wistar rats were given 4,4'-MDA orally in dose ranges of 1-12 mg/kg bw or injected i.p. with ^{14}C -4,4'-MDA at 25 mg/kg bw to study the binding of 4,4'-MDA to hemoglobin (17). Twenty-four hours after dosing, the two hemoglobin adducts MDA and *N*-acetyl-MDA were detected and accounted for 36 and 45% of the total radioactivity bound to the protein. A dose-response relationship was established in orally treated rats between production of each of the two adducts and the dose of 4,4'-MDA.

4,4'-MDA was also detected in DNA adducts in the liver 24 hours after *in vivo* exposure of Wistar rats (18). However, the major adducts did not correspond to synthesized standards of C-8 guanine adducts as analyzed by ^{32}P -postlabeling, HPLC or GC-MS, therefore, the adducts were not identified.

Irritation and sensitization

4,4'-MDA is moderately irritating for eyes (4, 19). 4,4'-MDA dihydrochloride is reported as minimally irritating to the respiratory system, but painfully irritating to the eyes (4). 4,4'-MDA can cause skin sensitization in humans, as shown by positive reactions on challenge in patch tests (20, 21).

Acute/subacute toxicity

Humans

4,4'-MDA is a causative agent in "Epping jaundice", which followed the accidental contamination of flour used for baking bread (22, 23). The 84 persons affected had symptoms including jaundice, tender liver, weakness, abdominal pain, nausea, vomiting, headache, fever, chills and muscle pain, as well as elevated levels of bilirubin, alkaline phosphatase and aspartate aminotransferase. Biopsies from 7 of the patients showed portal inflammation, eosinophil infiltration, cholangitis, cholestasis and different degrees of hepatocellular damage. All patients made good clinical recovery. The dose of 4,4'-MDA received was estimated to be about 3 mg/kg bw.

Occupational exposure to 4,4'-MDA, possibly through the skin, caused toxic hepatitis, experienced as acute febrile illness associated with jaundice, in 12 workers, all of whom recovered within seven weeks (24). Follow-up more than five years later showed no biochemical or clinical evidence of chronic hepatic disease. A skin rash was seen in five of the cases. Another case of hepatitis developed in a worker being in contact with 4,4'-MDA in another factory.

A case of acute myocardopathy, as well as toxic hepatitis, has been reported in a worker after a massive acute exposure to 4,4'-MDA at a chemical plant (25).

Animals

4,4'-MDA has moderately acute toxicity from oral exposure, with reported oral LD50 values of 120-830 mg/kg bw for rats, and 264, 620, 260 and 300 mg/kg bw for mice, rabbits, guinea pigs and dogs, respectively (4, 19).

Various short-term exposures of laboratory animals to 4,4'-MDA mostly affected the liver and bile duct, and sometimes also the kidneys, spleen and other organs (4, 6).

Male albino rats were given 4,4'-MDA by stomach tube once in doses of 50, 200, 250 and 600 mg/kg bw (acute exposure), or 8 times during 10 days in doses of 8, 20 and 50 mg/kg bw (subacute exposure), with or without the additional stress of heat (26). The substance induced liver enlargement and caused necrotizing cholangitis; in high doses (200-600 mg/kg bw) it induced small parportal necroses of liver parenchyma and a considerable loss of glycogen. It also caused marked mitotic activity, especially in doses of 20 and 50 mg/kg bw, associated with an increased rate of labeled thymidine in hepatocyte nuclei and bile duct epithelia. A significant decrease in the activity of succinic dehydrogenase, reduced nicotinamide adenine dinucleotide tetrazolium reductase, lactate dehydrogenase and non-specific acid phosphatase, and a significant increase in reaction of glucose-6-phosphate-dehydrogenase and non-specific alkaline phosphatase, and dose- and time-dependent alterations of glucose-6-phosphatase were observed. Moderate fatty degeneration of liver cells occurred. In several animals, high doses also caused damage to kidneys, heart muscle, brain and testicles, resulting in edema and parenchymatous degenerations. Additional heat stress did not cause any essential changes in the effect of 4,4'-MDA.

F344/N rats and B6C3F₁ mice received 4,4'-MDA as the dihydrochloride in drinking water for 14 days (27). Five rats/sex/group received 0, 200, 400, 800, 1600 and 3200 ppm, equivalent to 0, 18, 33, 37, 78 and 89 mg/kg bw/day in male rats, and 0, 17, 33, 51, 80 and 128 mg/kg bw/day in female rats, calculated on an assumed water intake of 100 g/kg bw/day, taking into account the drastic reduction in water consume, up to 72% in males and 60% in females. Five mice/sex/group received the same 4,4'-MDA concentrations, equivalent to 0, 32, 78, 136, 170 and 101 mg/kg bw/day in male mice, and 0, 30, 57, 102, 132 and 101 mg/kg bw/day in female mice, calculated on an assumed water intake of 150 g/kg bw/day, taking into account the reduced water consume, up to 79% in both sexes. Water consumption was lowered in all dosed rat groups and in male mice that received 1600 ppm or more, and in female mice at 800 ppm or higher. Mean body weight gain was depressed dose-dependent in all rat groups and in mice that received 800 ppm or more. In some rats receiving 1600 or 3200 ppm, crater-like foci with black content in the cardiac part of the stomach were noted. No premature deaths occurred in rats. Survival was reduced in some mice at 800 ppm or higher, all mice died at 3200 ppm. No compound-related lesions were identified in mice at necropsy. Hematology, clinical biochemistry and histopathological examinations were not performed in any species. A NOAEL was not determined in the rat study, whereas in mice the NOAEL was 400 ppm, equivalent to 78 and 57 mg/kg bw/day in males and females, respectively.

4,4'-MDA has been found to cause blindness in cats. A single near-lethal dose of 100 mg/kg produced selective atrophy of rods, cones and nuclei in outer granular layer (28).

Subchronic/chronic toxicity

Humans

No subchronic or chronic toxicity studies were available for effects of 4,4'-MDA in humans.

Animals

Subchronic (6 weeks) and chronic (16 weeks) oral administration of 8 mg/kg 4,4'-MDA to male albino rats caused minor liver damage, while 20 mg/kg caused manifest liver damage, which resulted in formation of hyperplastic nodules, adenoma-like bile duct proliferation and cirrhosis-like changes. In two animals, it caused hemangiomas of the liver (29).

F344/N rats and B6C3F₁ mice received 4,4'-MDA as the dihydrochloride in drinking water for 90 days (27). Ten rats/sex/group received 0, 50, 100, 200, 400 and 800 ppm, equivalent to 0, 4, 7, 13, 26 and 39 mg/kg bw/day in male rats, and 0, 4, 7, 13, 20 and 44 mg/kg bw/day in female rats. Ten mice/sex/group received 0, 25, 50, 100, 200 and 400 ppm, equivalent to 0, 3, 6, 11, 27 and 55 mg/kg bw/day in male mice, and 0, 4, 8, 14, 26 and 52 mg/kg bw/day in female mice. Hematology and clinical biochemistry were not performed in any species, but full histopathological examination of control animals and high dose animals was done. Liver, pituitary and thyroid of rats receiving 400 ppm, and liver and thyroid of rats receiving 200 ppm were also examined histologically. No animals died. The mean final body weight was depressed in male rats receiving 800 ppm and in female rats receiving 400 ppm. Water consumption was depressed 10% or more in both sexes of rats receiving 200 ppm or more. Bile duct hyperplasia and adenomatous goiter were observed dose-dependently in rats getting 400 and 800 ppm. Some rats receiving 400 ppm, and one male rat in the 800 ppm group, developed thyroid follicular hyperplasia. In all male rats, and 5/9 female rats getting 800 ppm, a pituitary basophil hypertrophy was found. In mice, mean body weight was depressed in males at 200 ppm and in females at 400 ppm. Water consumption of dosed male mice was greater than that in controls, and in female mice it was comparable to in the controls. Bile duct hyperplasia was found in 5/10 male mice and in 4/10 female mice that received 400 ppm. Adenomatous goiters, less severe than that observed in high dose rats, were observed in one high dose male and female mouse. The NOAEL in both studies was identified to be 100 ppm, equivalent to 7 and 7 mg/kg bw/day in male and female rats, respectively, and 11 and 14 mg/kg bw/day in male and female mice, respectively.

Non-neoplastic end points were reported from a carcinogenicity study (neoplastic results are reported below) where F344/N rats and B6C3F₁ mice received 4,4'-MDA as the dihydrochloride in drinking water for 2 years (27, 30, 31). Rats and mice were treated with 150 and 300 ppm 4,4'-MDA, equivalent to 9 and 16 mg/kg bw/day for male rats, and 10 and 19 mg/kg bw/day for female rats, 25 and 57 mg/kg bw/day for male mice, and 19 and 43 mg/kg bw/day for female mice. All animals showed increased incidences of non-neoplastic liver changes. Non-neoplastic lesions observed at the end of the treatment including unspecified dilatation (males only), fatty metamorphosis and focal cellular change were observed in rats of each dose groups, without a clear dose-response relationship. Liver cell degeneration was evident in most male mice of both dose groups and in 7/50 high dose females. Increases in cystic and hyperplastic follicular cell changes were seen in the thyroid of the rats, follicular cell hyperplasia occurred in mice. In both species, thyroid effects were evident with a slightly higher frequency in the low dose groups of male and female rats, and of male mice, compared to the control groups. Alterations occurred more frequently in all high dose groups of each species. Mineralization of the kidney was seen in increased incidences in high dose male rats. In male and female mice, higher incidences of renal nephropathy in the low and high dose groups, and papillary mineralisation in high dose group, compared with the controls, were observed. A clear dose-response relationship in the liver and thyroid changes was lacking. However, this may be explainable due to simultaneous or overlapping processes of degeneration/preneoplastic changes and tumour growth. The

LOAEL for non-neoplastic lesions, derived from toxic liver effects, was estimated to be 150 ppm in rats and mice, equivalent to 9 and 10 mg/kg bw/day in male and female rats, respectively, and 25 and 19 mg/kg bw/day in male and female mice, respectively. A NOAEL was not estimated from this study.

Developmental and reproductive toxicity

Humans

No data was available on reproductive or developmental effects of 4,4'-MDA in humans.

Animals

An acute duration study reported hypertrophy and a 71% increase in absolute weight of the uterus in ovariectomized female Sprague-Dawley rats given 150-200 mg/kg bw 4,4'-MDA dihydrochloride by gavage for 5-14 days (32). The adrenal and thyroid glands showed also hypertrophy and increased weight. Histopathological examination of the uterus revealed an atypical folliculoid response in the endometrium. No further information was provided in this study.

Very few and inadequately performed and reported studies of developmental and reproductive toxicity of 4,4'-MDA in animals are found. Fetuses from pregnant rats treated by gavage with 37 mg/kg bw/day 4,4'-MDA (as the chlorohydrate) on gestation days 14-20 had liver alterations in the form of fatty infiltration of the parenchyma (33). This dose level also caused histological alterations in the livers of the dams. Fetuses from 1 of 5 dams given 219 mg/kg bw/day 4,4'-MDA on gestation days 7-20 showed delayed closing of the calvaria, enlarged tongue, and an abnormally large snout. This dose was lethal to 1 of the 5 pregnant rats. Only one female rat was used as control.

No gross or histopathological alterations were observed in the ovaries, uterus, mammary glands, seminal vesicles, prostate or testes of rats given up to 44 mg/kg/day 4,4'-MDA dihydrochloride in drinking water for 90 days, or up to 19 mg/kg/day for 2 years (27). Similar results were reported in mice given up to 55 mg/kg/day 4,4'-MDA dihydrochloride in drinking water for 90 days or up to 57 mg/kg/day for 2 years (27). No further reproductive parameters were evaluated.

The limited information available is insufficient to determine whether exposure to 4,4'-MDA may affect development or alter reproductive function in animals.

Carcinogenicity

Humans

In a Swedish retrospective study (34), the state of health was investigated in three subgroups of power generator workers; 192 workers were exposed to 4,4'-MDA between 1963 and 1985, 237 workers were possibly exposed and 121 workers were unexposed. The concentration of 4,4'-MDA, the exposure route and time of exposure were not registered. No cancer cases occurred in the exposed group, two cancer cases occurred in the possibly exposed group, and four cases, included one case of bladder cancer, occurred in the unexposed group. The authors concluded that there was no statistically significant evidence of an increased overall or bladder cancer risk compared to the total population.

Ten workers exposed to 4,4'-MDA from 7 days to 2.5 months between 1967 and 1976 were followed (35). The concentration supposed to be inhaled ranged from 0.04-3.11 mg/m³. After developing acute jaundice the workers left the factory. Twenty-three years after intoxication, one worker was diagnosed with bladder cancer. Since the average latency period for aromatic amine-induced cancer has been suggested to be about 20 years, occurrence of bladder cancer has been observed in other persons occupationally exposed to 4,4'-MDA, and on the basis of animal data, the authors concluded that this finding added weight to the suggestion that 4,4'-MDA is carcinogenic in humans.

A follow-up investigation of 179 white male deaths among employees with potential exposure to epoxy resins and amine hardeners who had ever worked for more than one month in areas with potential exposure to 4,4'-MDA was conducted (36). Forty-six persons of this group died with malign neoplasms. The proportional mortality rate amongst these persons revealed a statistically significant excess of cancer of the large intestine, cancer of the bladder, lymphosarcoma and reticulosarcoma compared to the whole population. In a proportional cancer mortality ratio analysis the excess of bladder cancer remained significantly elevated. On the basis of these findings, the U.S. National Institute for Occupational Safety and Health (NIOSH) suggested an association between bladder cancer and work in areas with past or present potential exposure to 4,4'-MDA.

The results of a morbidity study of gaseous diffusion plant workers who had been potentially exposed to epoxy resins and hardeners including 4,4'-MDA, were reported (37). Five cases of bladder cancer were reported among 263 potentially exposed centrifuge workers and none in a control group of 271 persons, which is a 7.8 times greater incidence, and statistically significant. None of the five workers had routinely handled the epoxy materials, but had opportunity to pass through the area where the material was handled, helped with decontamination and clean-up, or were in close proximity to centrifuge operations.

These reports of limited reliability describing effects after repeated exposures of humans to 4,4'-MDA showed a coincidence of bladder cancer and work areas with exposure to 4,4'-MDA. However, since the route of exposure was not likely to be mainly oral, the dose and exposure time were unknown, and no data on confounding factors or exposure to other substances were given, these studies can not be used in quantitative risk assessment of 4,4'-MDA.

Animals

4,4'-MDA was administered to male rats (strain and age unspecified) at near the maximum tolerated dose by gastric intubation in arachis oil 5 days per week for 121 days (total dose 3.3 g/kg bw) (38). Of 24 male rats originally treated, 19 were alive at 12 months, 17 at 18 months, and 12 at 2 years. All animals had cirrhosis of the liver, but no tumours were found before 2 years. Two rats killed at 792 and 947 days respectively, had benign hepatomas. A variety of miscellaneous tumours was found in rats dying between 2 and 3 years. In a second experiment, intragastric dosing was continued for about 18 months (total dose 6 g/kg bw). Two liver tumours, 1 intestinal tumour, 1 pituitary tumour and 2 subcutaneous fibromas were found.

A pilot study was reported where groups of 8 male and 8 female rats (strain and age unspecified) received 4 or 5 doses of 20 mg/rat 4,4'-MDA (purity not stated) by gastric intubation over a period of less than 8 months, and were observed until death (39). One hepatoma and a hemangioma-like tumour of the kidney were found in a male rat after 18

months. An adenocarcinoma of the uterus was found in one female after 24 months. Most animals had varying degrees of liver fibrosis and inflammation.

Twenty female Sprague-Dawley rats, 40 days old, received 30 mg (maximum tolerated dose) 4,4'-MDA dihydrochloride (purity unspecified) in 1 ml sesame oil by gastric intubation every three days for 30 days (total dose, 300 mg/rat), and were observed for further nine months (40). A group of 140 female rats receiving sesame oil alone served as negative controls, and a group of 40 females receiving single doses of 18 mg 7,12-dimethylbenz[*a*]anthracene (DMBA) served as positive controls. Survival after nine months was 14/20 (70%) in the 4,4'-MDA-treated group, 127/140 (91%) in the negative-control group and 19/40 (48%) in the DMBA-treated group. Mammary lesions were found in 5/132 (4%) negative control rats (3 carcinomas, 1 fibroadenoma, 5 hyperplasias), 29/29 (100%) DMBA-treated rats (75 carcinomas, 10 fibroadenomas, 47 hyperplasias), and 1/14 (7%) 4,4'-MDA-treated rats (one hyperplasia).

In a study investigating the possible promoting activity of 4,4'-MDA on the development of thyroid tumours in rats treated with *N*-bis(2-hydroxypropyl)-nitrosamine (DHPN), 21 male inbred W rats, seven weeks old, received a single i.p. injection of 2800 mg/kg bw DHPN (purity unspecified), and were maintained on a diet containing 1000 mg/kg 4,4'-MDA (purity unspecified) for 19 weeks (41). In a second group, 21 males were fed a diet containing 1000 mg/kg diet 4,4'-MDA. Groups of 21 rats each received a single i.p. injection of either 2800 mg/kg bw DHPN (positive controls) or 0.5 ml saline/100 g bw (negative controls), followed by basal diet for 19 weeks. All animals were still alive at the termination of the experiment. There was a significant ($p < 0.05$) increase in the incidence of thyroid tumours (polymorphofollicular, microfollicular and papillary adenomas, and follicular carcinomas) in the rats treated with DHPN plus 4,4'-MDA (19/21, 90%) over that in the group treated with DHPN alone (6/21, 29%). 4,4'-MDA alone induced no thyroid or other tumours. In contrast to this study, in several other studies 4,4'-MDA reduced tumour incidences in various organs of rats when given at the post-initiation stage compared to in rats given only initiating carcinogens (examples given in 8). In these studies, 4,4'-MDA alone was not carcinogenic.

Groups of 50 male and 50 female 6-week old Fischer 344/N rats were given 150 ppm or 300 ppm 4,4'-MDA dihydrochloride (98.6% pure, dose expressed as the free base) in the drinking water for 103 weeks, followed by one week without treatment (27, 30, 31). The doses were equivalent to 9 and 16 mg/kg bw/day for male rats, and 10 and 19 mg/kg bw/day for female rats. Groups of 50 male and 50 female mice receiving drinking water adjusted with 0.1 *N* HCl to pH 3.7 (similar to pH in 300 ppm 4,4'-MDA) served as controls. There was no significant effect on survival in males or females. Mean body weight was reduced in high dose female rats. Water consumption was reduced in a dose-related manner in both sexes of rats. No compound-related clinical effects were observed. The incidence of thyroid follicular cell carcinomas in high dose males was significantly increased over that in controls; 0/49 control, 0/47 low dose and 7/48 high dose males (15%, $P < 0.012$). The incidence of thyroid follicular cell adenomas in high dose females was significantly increased over that in controls; 0/47 control, 2/47 (4%) low dose and 17/48 high dose females (35%, $P < 0.001$). Thyroid C-cell adenomas were also elevated in a dose-related manner in female rats; 0/47 controls, 3/47 (6%) low dose, 6/48 (13%, $P < 0.029$) high dose. A significant increase in the incidence of liver neoplastic nodules was also observed in male rats; 1/50 (2%) controls, 12/50 low dose (24%, $P = 0.002$) and 25/50 high dose (50%, $P < 0.001$) animals, and a statistically non-significant increase in these lesions was seen in treated females; 4/50 (8%) controls, 8/50 (16%) low dose and 8/50 (16%) high dose rats.

Groups of 50 male and 50 female 12-week old B6C3F₁ mice were given 150 ppm or 300 ppm 4,4'-MDA dihydrochloride (98.6% pure, dose expressed as the free base) in the drinking water for 103 weeks, followed by one week without treatment (27, 30, 31). The doses were equivalent to 25 and 57 mg/kg bw/day for male mice, and 19 and 43 mg/kg bw/day for female mice. Groups of 50 male and 50 female mice receiving drinking water adjusted with 0.1 N HCl to pH 3.7 (similar to pH in 300 ppm 4,4'-MDA) served as controls. Survival at termination was 40/50 (80%) control, 39/50 (78%) low dose and 32/50 (64%, $P=0.006$) high dose males, and 40/50 (80%) control, 38/50 (76%) low dose and 37/50 (74%) high dose females. Mean body weight was reduced in high dose male and female mice. No compound-related clinical effects were observed. An increased incidence of follicular cell adenomas of the thyroid was observed in high dose animals; 0/47 control, 3/49 (6%) low dose and 16/49 (33%, $P<0.001$) high dose males, and 0/50 control, 1/47 (2%) low dose and 13/50 (26%, $P<0.001$) high dose females. An increased incidence of hepatocellular adenomas occurred in females; 3/50 (6%) controls, 9/50 (18%) low dose and 12/50 (24%) high dose animals ($P\leq 0.01$). Increased incidences of hepatocellular carcinomas were observed in treated male mice; 10/49 (20%) controls, 33/50 (66%, $P<0.001$) low dose and 29/50 (58%, $P<0.001$) high dose animals, and in high dose female mice; 1/50 (2%) controls, 6/50 (12%) low dose, 11/50 (22%, $P=0.002$). Neoplasms were also seen in other organs, but then in one gender only. Pheochromocytomas in the adrenal gland medulla were observed in male mice; 2/48 (4%) controls, 12/49 (24%, $P<0.006$) low dose, 14/49 (29%, $P<0.05$) high dose. Lymphomas were observed in female mice; 13/50 (26%) controls, 28/50 (56%, $P=0.002$) low dose, 29/50 (58%, $P=0.001$) high dose. Alveolar/bronchiolar adenomas were observed in female mice; 1/50 (2%) controls, 2/50 (4%) low dose, 6/49 (12%, $P<0.05$) high dose.

A group of 5 female pure-bred beagle dogs, 5-6 months old, received oral administration of 70 mg 4,4'-MDA ("highly purified", dissolved in corn oil and placed in gelatinous capsules) thrice weekly (42). A further 4 female beagles received capsules of "crude" 4,4'-MDA (50% 4,4'-MDA, 50% higher MW analogs). Total doses were 5.0-6.26 g/kg bw "pure" 4,4'-MDA over periods of 4.5-7 years, at which time there was one survivor, and 4.0-6.25 mg/kg bw "crude" 4,4'-MDA over periods of 4-7 years, at which time there were two survivors. No tumours of the urinary bladder or liver were found. However, one tumour in the uterine horn and one in the spleen were noted, which were not examined microscopically. Moderate to severe gross and micropathological changes were observed in the liver, and less severe changes in the kidneys and spleen.

In conclusion, the results from the reports on human exposure did not clearly show a carcinogenic activity in humans. 4,4'-MDA is clearly carcinogenic in experimental animals, since two-year animal studies showed that oral treatment with 4,4'-MDA was associated with tumours of the thyroid and the liver in rats and mice. Therefore, animal data was used in the following risk characterization.

Genotoxicity

In vitro

4,4'-MDA was evaluated to be positive when tested for *in vitro* mutagenicity in *Salmonella typhimurium* strains TA100 and TA98 with metabolic activation, but was negative without activation (4, 43), and negative in *Salmonella typhimurium* strains TA1535, TA1537, TA1538 and *Saccharomyces cerevisiae* strain D4 in the absence or presence of metabolic activation by rat, mouse or monkey liver S9 (4). However, one study reported a weak positive response also

in *Salmonella typhimurium* strain 1538, as well as in TA98, in the presence of rat liver S9 (4). 4,4'-MDA dihydrochloride was evaluated to be positive when tested for *in vitro* mutagenicity in *Salmonella typhimurium* strains TA100, TA98, TA97 and TA 1535 with metabolic activation, but was negative without activation (43, 44), and negative in *Escherichia coli* WP2 UVRA with or without metabolic activation (45).

In contrast to MDA, the acetylated metabolites *N*-acetyl-MDA and *N,N'*-diacetyl-MDA were not mutagenic in *Salmonella typhimurium* TA 98 or TA 100 with or without metabolic activation by rat liver S9 (15).

4,4'-MDA dihydrochloride was positive in the *in vitro* L5178Y tk⁺/tk⁻ mouse lymphoma cell forward mutation assay without metabolic activation (43, 46).

4,4'-MDA failed to induce chromosomal aberrations or sister chromatid exchange in human leucocytes *in vitro* either in the presence or absence of rat liver microsomal enzymes (47, 48).

4,4'-MDA was positive for *in vitro* sister chromatid exchanges with and without metabolic activation in CHO cells, but negative for *in vitro* chromosome aberrations with and without metabolic activation in CHO cells (43). 4,4'-MDA dihydrochloride was positive for *in vitro* sister chromatid exchanges and for chromosome aberrations with and without metabolic activation in CHO cells (43, 49).

A statistically significant dose-dependent increased frequency of micronuclei was observed after *in vitro* exposure of Chinese hamster lung fibroblasts (V79) cells to 50-500 µg/ml 4,4'-MDA, both with and without metabolic activation (50). 4,4'-MDA-induced micronuclei formation was demonstrated to be due to induction of clastogenic chromosomal damage (51).

4,4'-MDA was evaluated to be clearly positive when tested in untreated ACI/N rat hepatocyte primary culture/DNA repair test, which measure unscheduled DNA synthesis (UDS) *in vitro* (52). 4,4'-MDA increased to a marginal extent DNA repair synthesis (unscheduled DNA synthesis) in primary male Sprague-Dawley rat hepatocytes after pretreatment of the rats with Arochlor 1254 or phenobarbital, but not in hepatocytes from untreated rats (53).

After exposure for 4 and 20 hours to 4,4'-MDA concentrations ranging from 10 to 180 µM, a statistically significant increase in the frequency of DNA lesions was revealed by the Comet assay in primary hepatocytes and thyrocytes from humans and rats (54). DNA was damaged to a lesser extent in human hepatocytes and thyrocytes than in corresponding rat cells, and in both species in hepatocytes compared with in thyrocytes. DNA damage was absent in primary kidney and urinary bladder cells from humans. 4,4'-MDA did not induce DNA damage in primary cells from rat kidney, urinary bladder mucosa or brain, which are resistant to 4,4'-MDA carcinogenesis. The authors conclude that the results as a whole indicate that 4,4'-MDA is specifically activated to DNA-damaging reactive species by hepatocytes and thyrocytes in both humans and rats, and thus suggest that liver and thyroid might be the target of the carcinogenic activity of 4,4'-MDA also in humans.

Alkaline elution of DNA showed that *in vitro* exposure of Chinese hamster lung fibroblasts (V79) cells to 4,4'-MDA (1-3 mM) induced DNA damage in the presence of rat liver S9 (55).

In vivo

4,4'-MDA was positive in *in vivo* micronucleus test, sister chromatid exchanges and chromosome aberrations in male B6C3F1 mice (43).

Male Swiss mice received 9 and 18 mg/kg bw 4,4'-MDA (98% purity) by i.p. injection (56). The incidence of sister chromatid exchange in femoral bone marrow cells was determined 24 hours after treatment in the presence of 5-bromodeoxyuridine. Both dose levels induced statistically significant increases of 1.35- and 1.39-fold, for the low and high dose, respectively.

A statistically significant dose-related increase in sister chromatid exchange was observed in bone-marrow cells recovered from BALB/c mice treated with 1-35 mg/kg bw 4,4'-MDA, a doubling in frequency being seen with 35 mg/kg bw (57).

In two experiments with 5 male B6C3F1 mice per group, 3 daily i.p. injections of 9.3, 18.5 and 37.0 mg/kg bw 4,4'-MDA dihydrochloride led to increased bone marrow micronucleus frequencies, which were less than 2-fold (0.23 to 0.35%) compared to the negative controls (0.17 and 0.19%) (58). The micronucleus frequencies in the treated animals were within the range of negative control values obtained for the 49 chemicals tested in this investigation.

In experiments using peripheral blood from male CD-1 mice (5 per group), with single i.p. injections, a weak, but dose-dependent increase in micronucleated reticulocytes was observed in one experiment (doses 28 to 112 mg/kg bw), and a marginal increase in another experiment (28 to 140 mg/kg bw), after 48 hours (59). Two daily doses ranging from 22.5 to 90.0 mg/kg bw were negative. The overall results were regarded as inconclusive.

4,4'-MDA failed to induce a positive response in the *in vivo* - *in vitro* Fischer-344 rat or B6C3F1 mouse hepatocyte DNA repair assay, when no pretreatment was used to induce hepatic enzymes (60). In this study, oral doses given by gavage were up to 80% of the LD50, i.e. 20, 80 and 350 mg/kg bw for rats, and 50, 200, 500 and 1000 mg/kg bw for mice, and the sampling times were 2 and 12 hours after treatment. However, 4,4'-MDA was a potent inducer of S-phase synthesis in the rat liver.

I.p. injection of 74 mg/kg bw 4,4'-MDA into male Sprague-Dawley rats clearly induced DNA damage, as increased DNA fragmentation detected by alkaline elution analysis in the liver 4 and 24 hours after injection (61). Since elution was run under pH 12.3, primarily single and double strand breaks in DNA were detected, not alkali-labile sites.

A single oral gavage of 250 mg/kg bw 4,4'-MDA to male ddY mice gave statistically significant responses in stomach, liver, kidney, bladder, lung and brain after 8 and/or 24 hours measured by the alkaline single cell gel electrophoresis assay, which detects DNA damage (62).

4,4'-MDA induced mutation in yeast in the presence and absence of metabolic activation, but failed to induce sex-linked recessive lethal mutations in *Drosophila melanogaster* (47). However, 4,4'-MDA dihydrochloride did induce sex-linked recessive lethal mutations in postmeiotic and meiotic germ cells of male *Drosophila melanogaster*, but did not induce reciprocal translocations (43, 63).

In conclusion, there is insufficient evidence to indicate that exposure to 4,4'-MDA causes genetic damage in humans, whereas it is mutagenic and genotoxic in microorganisms and experimental animals.

Classification

4,4'-MDA was found to be genotoxic *in vitro* and *in vivo*. The reports on human exposure to 4,4'-MDA did not show clearly the presence of carcinogenic activity in humans, whereas 4,4'-MDA was found to be carcinogenic in both rats and mice (27, 30, 31). The mechanisms of 4,4'-MDA carcinogenicity are not yet known. In the absence of evidence that the liver and thyroid tumours observed in rats and mice are a consequence of chronic tissue-damaging (liver) or tissue-stimulating (thyroid) effects, it is prudent to assume that a genotoxic mechanism is involved, and that 4,4'-MDA is a non-threshold carcinogen.

According to EU classification, 4,4'-MDA is classified as a category 3 mutagen (substances which cause concern for man owing to possible mutagenic effects), and as a category 2 carcinogen (substances which should be regarded as if they are carcinogenic to man) (64). 4,4'-MDA is classified by IARC in group 2B - possible carcinogenic to humans, based on sufficient evidence for carcinogenicity in rats and mice (65). The U.S. Environmental Protection Agency (EPA) considers 4,4'-MDA as a suspected human carcinogen (66).

4,4'-MDA is on the Norwegian environmental authorities' list of substances with adverse effects on health or environment that may represent particular problems in Norway ("Obs-listen"), because of its chronic toxicity and carcinogenicity (67).

Determination of dose descriptors for carcinogenicity

Because 4,4'-MDA is genotoxic and a non-threshold carcinogen, a dose descriptor needs to be determined as point of comparison on the dose-response curve for the subsequent risk characterization.

The EFSA Scientific Committee proposed to use the Benchmark Dose Lower Limit (BMDL) corresponding to the lower limit of an one-sided 95% confidence limit interval on the Benchmark dose (BMD) corresponding to a 10% tumour incidence above the control (BMDL10) as a dose descriptor in risk assessment of chemicals that are both genotoxic and carcinogenic (68).

Carcinogenicity has to be considered the critical human end point after exposure to 4,4'-MDA. In the best reported and documented carcinogenicity experiment, 4,4'-MDA dihydrochloride was given in drinking water for 2 years (27, 30, 31). In F344/N rats, treatment-related increases in the incidences of thyroid follicular cell carcinomas and neoplastic hepatic nodules were observed in males, and thyroid follicular cell adenomas and thyroid C-cell adenomas occurred in females. In B6C3F₁ mice, treatment-related increases in the incidences of thyroid follicular cell adenomas and hepatocellular carcinomas were found in both males and females, adenomas of the liver and lymphomas in female mice, and adrenal pheochromocytomas in male mice.

The most sensitive end point in these experiments was incidence of neoplastic hepatic nodules in male rats, where both treatment doses increased the incidences significantly compared with the control group. The incidences of neoplastic nodules in the liver of male rats were 1/50

(2%), 12/50 (24%) and 25/50 (50%) in the negative control, 150 ppm (9 mg/kg bw/day) and 300 ppm (16 mg/kg bw/day) 4,4'-MDA groups, respectively, giving net values of 22% and 48% incidences in the low and high dose groups, respectively (27, 30, 31). Based on these data and using a multistage model with extra risk in the U.S. EPA Benchmark Dose Software (69), a BMDL10 of 2.33 mg/kg bw/day for the 4,4'-MDA dihydrochloride was calculated, or 1.7 mg/kg bw/day, for the 4,4'-MDA base.

An alternative dose descriptor is T25, the dose corresponding to a 25% tumour incidence, corrected for spontaneous tumours. There have been shown good correlations between the T25 and the carcinogenic potency index TD50 (70), epidemiological studies (71) and the lowest effective dose (LED) giving a response in genotoxicity tests *in vivo* (72).

We also calculated T25 as an alternative dose descriptor, using the same end point as above (27, 30, 31). Both doses increased the incidences of neoplastic nodules in the liver of male rats significantly, however, the 300 ppm dose gave the lowest T25 value; $16 \text{ mg/kg bw/day} \times 25\%/48\% = 8.33 \text{ mg/kg bw/day}$ for the 4,4'-MDA dihydrochloride, or 6.1 mg/kg bw/day, calculated for the 4,4'-MDA base.

Exposure characterization

In 2004, the Norwegian Food Safety Authority conducted a survey on 4,4'-MDA migrated from cooking utensils collected on the Norwegian market (1). Fifty different polyamide/nylon cooking utensils were tested with water simulants at 100 °C for 60 min. The test water samples were screened by GC-MS analysis. The migration levels were quantified by HPLC and confirmed by quantitative GC-MS. When tested repeatedly with water at 100 °C for time periods of 30 min., the third test period revealed two items that exceeded the limit for migration of primary aromatic amines, expressed as aniline (not detectable, i.e. 0.02 mg/kg food or food simulant, according to the EU plastics directive 2002/72/EC (2)), a balloon whisk with $1089 \mu\text{g}/\text{dm}^2$ 4,4'-MDA and a pasta spoon with $93 \mu\text{g}/\text{dm}^2$ 4,4'-MDA, quantitated with HPLC. The corresponding values quantitated by GC-MS were $1072 \mu\text{g}/\text{dm}^2$ and $113 \mu\text{g}/\text{dm}^2$, respectively. In addition, from a ladle tested with water at 100 °C for 60 min. followed by a second 30 min. test, a level of $42 \mu\text{g}/\text{dm}^2$ of 4,4'-MDA was quantitated with HPLC.

The highest concentration of 4,4'-MDA found was $1089 \mu\text{g}/\text{dm}^2$ migrated from a balloon whisk (1). In the following, intake of 4,4'-MDA from exposure to this cooking utensil has been estimated by assuming a surface area of the utensil of maximum 1 dm^2 used in a pot with a volume of 1 litre for 30 min., and that the whole food content cooked in this pot is consumed per day by one person with a body weight of 70 kg. In such a worst case situation, the exposure will represent a daily intake of $15.6 \mu\text{g}/\text{kg bw/day}$. Intake of 4,4'-MDA from other potential sources than cooking utensils was not taken into consideration in this risk assessment due to lack of Norwegian data.

By using a somewhat different scenario for the use of such kitchen utensils, Danish authorities have estimated a maximum daily intake of $1 \mu\text{g}/\text{kg bw/day}$ of 4,4'-MDA (73).

Risk characterization

The EFSA Scientific Committee has recommended using a margin of exposure (MOE) approach for quantitative risk assessment of substances that are both genotoxic and

carcinogenic (68). The MOE is the ratio between a defined point on the dose-response curve for the adverse effect, usually based on animal experiments in the absence of human data, and the estimated human intake. A MOE can be calculated by dividing the dose descriptor BMDL10 by the level of human exposure (E).

By using the BMDL10 calculated above and the estimated exposure, the $MOE = 1.7 \text{ mg/kg bw/day} / 15.6 \text{ } \mu\text{g/kg bw/day} = 109$

This MOE value is much lower than 10 000, which the EFSA Scientific Committee view as a low priority for risk management (68).

We also estimated by linear extrapolation the theoretical human cancer risk based on the T25 value of 6.1 mg/kg bw/day calculated above for rats. An equivalent value for humans (HT25), can be calculated using allometric scaling. The mean body weight throughout the experiment of the rats exposed to the high dose was 0.414 kg (27), and the human default body weight is 70 kg.

$$HT25 = T25 \text{ for rats (mg/kg bw/day)} \times (\text{kg bw for rats/kg bw for humans})^{0.25}$$

$$HT25 = 6.1 \text{ mg/kg bw/day} \times (0.414 \text{ kg bw}/70 \text{ kg bw})^{0.25} = 1.7 \text{ mg/kg bw/day}$$

$$HT100 \text{ (the whole population)} = 1.7 \text{ mg/kg bw/day}/0.25 = 6.8 \text{ mg/kg bw/day}$$

From a 4,4'-MDA exposure of 15.6 $\mu\text{g/kg bw/day}$, a human cancer risk can be calculated:

$0.0156 \text{ mg/kg bw/day} / 6.8 \text{ mg/kg bw/day} = 2.3 \times 10^{-3}$, which is a lifetime cancer risk which we consider to be of major concern.

Alternatively, by using the daily intake of 4,4'-MDA of 1 $\mu\text{g/kg bw/day}$ estimated by the Danish authorities (73), a MOE of 1700 is found, also being well below a MOE of 10 000. The human lifetime cancer risk based on T25 with this exposure level is 1.5×10^{-4} , which we also consider to be of major concern.

Also, based on the levels of up to 2067 $\mu\text{g/dm}^2$ 4,4'-MDA found elsewhere in Europe and reported as RASFF alerts (5), the conclusion is the same.

CONCLUSIONS

The highest concentration of 4,4'-MDA found to migrate from a cooking utensil collected from the Norwegian market was 1089 $\mu\text{g/dm}^2$. Based on this level of 4,4'-MDA and a worst case intake scenario, a human intake of 15.6 $\mu\text{g/kg bw/day}$ was estimated. 4,4'-MDA is genotoxic and carcinogenic in experimental animals, and is to be considered a non-threshold carcinogen. When the human cancer risk was evaluated by a margin of exposure (MOE) approach using the dose descriptor BMDL10, the MOE was only 109. Alternatively, a lifetime cancer risk for humans of 2.3×10^{-3} was quantitated using the calculated dose descriptor T25. Based on both of these values, we consider the level of migration of 4,4'-MDA from these kitchen utensils to be of major concern. The same conclusion was reached by using an alternative exposure estimated by Danish authorities or levels of migration detected elsewhere in Europe.

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