3-CHLORO-4-(DICHLOROMETHYL)-5-HYDROXY-2(5*H*)-FURANONE (MX)

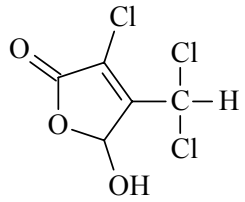
1. Exposure Data

1.1 Chemical and physical data

1.1.1 *Nomenclature*

Chem. Abstr. Serv. Reg. No.: 77439-76-0
Deleted CAS Reg. No.: 124054-17-7
Chem. Abstr. Name: 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone
IUPAC Systematic Name: 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone
Synonyms: Chloro(dichloromethyl)-5-hydroxy-2(5*H*)-furanone; MX

1.1.2 *Structural and molecular formulae and relative molecular mass*



$C_5H_3Cl_3O_3$ Relative molecular mass: 217.43

1.1.3 *Chemical and physical properties of the pure substance*

(*a*) *Description*: Pale yellow to brown viscous oil (Padmapriya *et al*., 1985; Sigma Chemical Co., 2002)
(*b*) *Boiling-point*: Not reported
(*c*) *Melting-point*: Not reported
(*d*) *Spectroscopy data*: Infrared, ultraviolet, nuclear magnetic resonance (proton, C-13) and mass spectral data have been reported (Padmapriya *et al*., 1985)

(*e*) *Solubility*: Soluble in water (50.8 mg/mL at pH 2; 43.7 mg/mL at pH 7); soluble in organic solvents (Vartiainen *et al*., 1991; Sigma Chemical Co., 2002)

(*f*) *Stability*: Stable in ethyl acetate and acidic water solutions (Vartiainen *et al*., 1991); may decompose on exposure to light or heat (Sigma Chemical Co., 2002)

(*g*) *Octanol/water partition coefficient (P)*: 1.13 at pH 2; –0.44 at pH 7 (Vartiainen *et al*., 1991)

1.1.4 *Technical products and impurities*

3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) is not known to be produced commercially except for research purposes (Sigma-Aldrich Co., 2002).

1.1.5 *Analysis*

Analysis in water samples is difficult because MX is usually present at trace levels (nanograms per litre) and it is thermolabile. Analytical methods for MX require pre-concentration of several litres of water, a clean-up of the water extract (adsorption on XAD resins, desorption with ethyl acetate, evaporation of the solvent), its derivatization (methylation with an acidic methanol solution) and high-resolution gas chromatography (GC) coupled to low- or high-resolution mass spectrometry (MS). An alternative to high-resolution MS is an ion-trap mass spectrometer with MS capabilities. An ion-trap detector with electron ionization and MS–MS fragmentation was used for the selective determination of MX and its chlorinated and brominated analogues (Charles *et al*., 1992; Romero *et al*., 1997; Onstad & Weinberg, 2001; Zwiener & Kronberg, 2001).

In potable water, MX is routinely determined by derivatization with methanol, followed by GC–MS. Electron capture detection is usually only suitable to detect MX present in clean matrices; chlorinated tap-water cannot be analysed for MX by this method due to the presence of many interfering contaminants. Derivatization with other alcohols can improve the detection of MX. Results achieved using propyl alcohols have been described. Results obtained with butyl alcohols as MX derivatization agents have also been reported; derivatization with *sec*-butanol was presented as a method which significantly lowers GC–MS detection levels of MX (Nawrocki *et al*., 1997, 2001).

1.2 Production and use

1.2.1 *Production*

Padmapriya *et al*. (1985) and Franzén and Kronberg (1995) have reported methods for the synthesis of MX.

MX is produced commercially only in small quantities for research purposes. It is a product of the chlorine-based disinfection of drinking-water (see Section 1.3.4). Like other

disinfection by-products, MX is formed at the first stage of the treatment process (prechlorination), then decreases in subsequent treatment stages, is totally removed in the carbon filters and finally may be formed again in the post-chlorination step (Romero *et al*., 1997).

MX and chlorinated acetic acids such as dichloroacetic acid and trichloroacetic acid have been the focus of a number of laboratory studies of disinfection by-products. To determine the effects of reaction time, total organic carbon, chlorine dose, pH and temperature on the formation of MX, dichloroacetic acid and trichloroacetic acid, fulvic acid was extracted from the sediment of Tai Lake (China), and simulated chlorination of samples rich in fulvic acid was conducted. Results showed a positive correlation between total organic carbon and the yields of MX, dichloroacetic acid and trichloroacetic acid. The influences of pH, chlorine dose, reaction time and temperature were quite complex. The optimal chlorination condition for the formation of MX was pH 2, a temperature of 45 °C, a C/Cl ratio of 1/4 and a reaction time of 12 h. Lower pH, a longer reaction time and a higher chlorine dose resulted in greater yields of both dichloroacetic acid and trichloroacetic acid; there was a strong linear relation between the formation of dichloroacetic acid and trichloroacetic acid, but there was no direct correlation between levels of the haloacetic acids and MX (Chen *et al*., 2001).

1.2.2 *Use*

MX has no known commercial uses.

1.3 Occurrence

1.3.1 *Natural occurrence*

MX is not known to occur as a natural product.

1.3.2 *Occupational exposure*

No data were available to the Working Group.

1.3.3 *Air*

No data were available to the Working Group.

1.3.4 *Water*

MX has been detected in water in Canada (Andrews *et al*., 1990), China (Zou *et al*., 1995), Finland (Kronberg & Vartiainen, 1988; Kronberg & Franzén, 1993; Kronberg, 1999), Japan (Suzuki & Nakanishi, 1990), Spain (Romero *et al*., 1997), the United Kingdom (Horth, 1990) and the USA (Meier *et al*., 1987a; Kronberg *et al*., 1991). Concentrations of MX in water are given in Table 1.

Table 1. Concentrations of MX in water

Water type (location)	Concentration range (ng/L)	Reference
Chlorinated raw water[a] (Finland)	280–510	Hemming *et al.* (1986)
Chlorinated raw water (Netherlands)	< 15	Backlund *et al*. (1989)
Treatment plant (United Kingdom)	< 3–41	Horth (1990)
Drinking-water (Australia)	< 0.5–33	Simpson & Hayes (1998)
Drinking-water (China)	3.8–58.4	Zou *et al.* (1995)
Drinking-water (Finland)	< 4–67	Kronberg & Vartiainen (1988)
Drinking-water (Japan)	< 3–9	Suzuki *&* Nakanishi (1990)
Drinking-water (USA)	2–33	Meier *et al.* (1987a)
Drinking-water (USA)	4.0–79.9	Wright *et al.* (2002)
Various waters including drinking-water (Spain)	0.1–56	Romero *et al.* (1997)

[a] Chlorinated raw water = water chlorinated in laboratory to estimate formation of MX

MX is produced as a by-product during disinfection of water containing humic substances using chlorine, chlorine dioxide or chloramines (Backlund *et al.,* 1988; Kanniganti *et al.*, 1992; Xu *et al.*, 1997). Consequently, it may occur in drinking-water after chlorine-based disinfection of raw waters containing natural organic substances. Before it was first synthesized in 1985 (Padmapriya *et al.,* 1985), it was referred to simply as MX (mutagen X). MX has been reported to account for between 15 and 60% of the mutagenicity in water based on the results of the *Salmonella* mutagenicity assay (Hemming *et al.*, 1986; Meier *et al.*, 1987a,b; Kronberg & Vartiainen, 1988; Horth *et al.*, 1989; Wright *et al.*, 2002). MX was initially detected in pulp chlorination waters in the early 1980s and subsequently in chlorinated drinking-waters (Holmbom *et al*., 1984; Hemming *et al*., 1986; Holmbom, 1990). MX in water gradually undergoes pH-dependent isomerization (to E-MX, 2-chloro-3-(dichloromethyl)-4-oxobutenoic acid) and hydrolytic degradation; at pH 8 and 23 °C, the half-life of MX is 6 days (Kronberg & Christman, 1989). In the USA, concentrations of MX increased with multiple chlorine applications, chlorine dose and total organic carbon, decreased with an increase in pH, were lower in chloraminated systems and showed seasonal variation with higher levels occurring in the spring compared with the autumn (Wright *et al*., 2002).

1.4 Regulations and guidelines

The WHO (1998) and Australia and New Zealand (National Health and Medical Research Council and Agriculture and Resource Management Council of Australia and New Zealand, 1996) note that insufficient data on health effects are available to establish a guideline for MX. The guideline in Australia and New Zealand also encourages a minimization of the concentration of all chlorination by-products by reducing naturally occur-

ring organic material from the source water, reducing the amount of chlorine added, or using an alternative disinfectant, while not compromising disinfection.

The European Union (European Commission, 1998) and Canada (Health Canada, 2003) have not set a guideline but encourage the reduction of concentrations of total disinfection by-products. The Environmental Protection Agency (1998) in the USA controls the formation of unregulated disinfection by-products, which would include MX, with regulatory requirements for the reduction of their precursors, in this case, total organic carbon. The Stage 1 Disinfectants/Disinfection By-Product Rule mandates a reduction in the percentage of total organic carbon between source water and finished water based on the original quantity in source water and alkalinity. Enhanced coagulation or enhanced softening are mandated as a treatment technique for this reduction, unless the levels of total organic carbon or disinfection by-products in source water are low.

2. Studies of Cancer in Humans

See Introduction to the monographs on chloramine, chloral and chloral hydrate, dichloroacetic acid, trichloroacetic acid and 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone.

3. Studies of Cancer in Experimental Animals

3.1 Oral administration

3.1.1 *Mouse*

Groups of 10 male and 10 female C57Bl/6J-*Min*$^{-/-}$ mice [age unspecified] were administered 0.20 mg/mL MX (94% pure dissolved at pH 3.4–4.0) in sterile drinking-water for 6 weeks; control groups received sterile water only. The animals were then given sterile water for an additional 4 weeks after which the animals were killed. Calculated daily doses of MX were 33 mg/kg bw for males and 42 mg/kg bw for females. The small intestine, colon and caecum were removed and processed for the measurement of aberrant crypt foci. For continuous data, the Student *t*-test or Mann-Whitney rank sum test was used for comparisons between the two treatment groups. The one-way ANOVA or Kruskal-Wallis ANOVA on ranks was used for comparisons among more than two groups. When a significance was noted, individual differences were tested by the Student-Newman-Keuls multiple comparison procedure. Differences between incidences of lesions were evaluated by the chi-square test or by Fisher's exact probability test (two-tailed). Male mice treated with MX had depressed water consumption (~8%) compared with the control value (3.5 ± 0.4 mL versus 3.8 ± 0.5 mL; $p = 0.006$). All mice had aberrant crypt foci ranging from 4 to 18 foci per animal, and MX in the drinking-water had no effect on aberrant crypt foci in the colon

or caecum [data not presented]. No significant differences among treatment groups were found for tumours in the small intestine (Table 2) or in the colon (Table 3) (Steffensen *et al.*, 1999).

Table 2. Incidence of tumours in the small intestine of C57Bl/6J-*Min*$^{-/-}$ mice after exposure to MX in the drinking-water

Sex	Treatment group	Incidence[a]	Number of tumours[b]	Diameter (mm)
Male	Water	10/10	41.9 ± 6.8	0.94 ± 0.11
	33 mg/kg bw MX	10/10	46.8 ± 7.9	0.98 ± 0.10
Female	Water	9/9	53.6 ± 11.5	0.97 ± 0.14
	42 mg/kg bw MX	10/10	57.3 ± 16.0	0.90 ± 0.16

From Steffensen *et al*. (1999)
[a] Number of mice with tumours/total number in group
[b] Mean ± standard deviation

Table 3. Incidence of tumours in the colon of C57Bl/6J-*Min*$^{-/-}$ mice after exposure to MX in the drinking-water

Sex	Treatment group	Incidence[a]	Number of tumours[b]	Diameter (mm)
Male	Water	7/10	1.0 ± 0.9	3.76 ± 1.49
	33 mg/kg bw MX	6/10	0.9 ± 1.0	3.41 ± 1.37
Female	Water	2/9	0.3 ± 0.7	3.25 ± 0.66
	42 mg/kg bw MX	4/10	0.5 ± 0.7	3.28 ± 1.12

From Steffensen *et al.* (1999)
[a] Number of mice with tumours/total number in group
[b] Mean ± standard deviation

3.1.2 *Rat*

Groups of 50 male and 50 female Wistar rats, 5 weeks of age, were exposed to MX (97% pure) in the drinking-water for 104 weeks. The MX solutions, prepared every 1–2 weeks, were adjusted to a pH range of 3.5–5.0. The concentrations of MX were set to yield doses that were at or below a no-observed-effect level to preclude overt toxicity (5.9 µg/mL, 18.7 µg/mL and 70.0 µg/mL), giving average daily doses of MX of 0.4 (low dose), 1.3 (mid dose) and 5.0 mg/kg bw (high dose) for males and 0.6 (low dose), 1.9 (mid dose) and

6.6 mg/kg bw (high dose) for females. Control animals were exposed to the vehicle as well as filtered and ultraviolet-irradiated tap-water. For all parametric data (e.g. body weight, water and feed consumption), the one-way analysis for variance was used to test for significant differences between groups. When differences were found, Dunnett's or Scheffe's test was used to determine significance between control and treatment groups. Mortality was analysed by the Kaplan-Meier method and survival curves by the log-rank test. Water consumption was depressed in mid- and high-dose males (5–20% decrease; $p < 0.01$) and females (6–24% decrease; $p < 0.05$). The decreased water consumption did not affect the water balance as reflected by no differences in urine volume among all treatment groups. No overt effects of MX on toxicity or mortality were found for either males or females. Body weight depression was observed only for high-dose males ($p < 0.01$) and females ($p < 0.05$). For any particular tumour site, the dose–response was analysed by the one-sided trend test. The findings from the study indicated that MX is a multisite carcinogen that induces both benign and malignant tumours in both male (see Table 4) and female rats (see Table 5). A dose-related statistically significant increase in the incidence of tumours was observed in the liver, thyroid gland, adrenal gland, lung and pancreas of males and in the liver, thyroid gland, adrenal gland and mammary gland and for lymphoma and leukaemia in females (Komulainen *et al.* 1997).

3.2 Administration with known carcinogens

3.2.1 *Mouse*

Groups of male BALB/cABOM mice [initial numbers unspecified], 4 weeks of age, were administered an intraperitoneal injection of 7 mg/kg bw azoxymethane or 0.9% sodium chloride (vehicle control) once a week for 2 weeks. One week later, animals were administered 0 or 20 mg/kg bw MX (94% pure dissolved in sterile water at pH 3.4–4.0) intrarectally three times weekly for 6 weeks to give a total MX dose of 360 mg/kg or 40 mg/kg bw MX three times weekly over 4 weeks, giving a total MX dose of 480 mg/kg. All mice were killed 15 weeks after the start of the experiment, 6 or 8 weeks after the last dose. The small intestine, colon and caecum were removed. The colon and caecum were scored for altered crypt foci and small intestine, colon and caecum were scored for tumours [for a description of statistical methods, see Section 3.1.1]. Neither dose of MX increased the incidence of aberrant crypt foci per colon when compared with sodium chloride or azoxymethane. MX (40 mg/kg) without prior initiation with azoxymethane increased the number of crypts per aberrant crypt focus when compared with the control value (Table 6). Most mice in every treatment group, including the vehicle control, had small tumours (0.3–0.6 mm, 1–16 per small intestine). One mouse receiving azoxymethane and 40 mg/kg bw MX had a colon tumour (Steffensen *et al.*, 1999). [The Working Group noted the inadequacy of the strain and dose of azoxymethane.]

Table 4. Incidence of primary tumours having a statistically significantly positive trend in male Wistar rats

Tissue site	Control	0.4 mg/kg bw per day MX	1.3 mg/kg bw per day MX	5.0 mg/kg bw per day MX	*p*-value trend test
Integumentary system					
Basal-cell tumour skin	1/50 (2%)	0/50	1/50 (2%)	3/50 (6%)	0.0314
Respiratory system					
Alveolar and bronchiolar adenoma	2/50 (4%)	1/50 (2%)	1/50 (2%)	7/50 (14%)	0.0015
Liver					
Cholangioma	0/50	0/50	1/50 (2%)	4/50 (8%)	0.0009
Adenoma	0/50	1/50 (2%)	2/50 (4%)	4/50 (8%)	0.0142
Pancreas					
Langerhans' cell adenoma	5/50 (10%)	8/50 (16%)	8/50 (16%)	12/50 (24%)	0.0116
Adrenal gland					
Cortical adenoma	5/50 (10%)	2/50 (4%)	7/50 (14%)	14/50 (28%)	0.0001
Thyroid gland					
Follicular carcinoma	0/49	1/50 (2%)	9/50 (18%)	27/49 (55%)	0.000
Follicular adenoma	2/49 (4%)	20/50 (40%)	34/50 (68%)	21/49 (43%)	0.0045

From Komulainen *et al*. (1997)

Table 5. Incidence of primary tumours having a statistically significantly positive trend in female Wistar rats

Tissue site	Control	0.6 mg/kg bw per day MX	1.9 mg/kg bw per day MX	6.6 mg/kg bw per day MX	*p*-value
Integumentary system					
Mammary gland					
Adenocarcinoma	3/50 (6%)	2/50 (4%)	5/50 (10%)	11/50 (22%)	0.0012
Fibroadenoma	23/50 (46%)	25/50 (50%)	32/50 (64%)	34/50 (68%)	0.0090
Haematopoietic system					
Lymphoma and leukaemia	1/50 (2%)	1/50 (2%)	2/50 (4%)	4/50 (8%)	0.0474
Liver					
Cholangioma	0/50	4/50 (8%)	10/50 (20%)	33/50 (66%)	0.0000
Adenoma	1/50 (2%)	1/50 (2%)	1/50 (2%)	10/50 (20%)	0.0000
Adrenal gland					
Cortical adenoma	5/50 (10%)	10/50 (20%)	12/50 (24%)	16/50 (32%)	0.0098
Thyroid gland					
Follicular carcinoma	1/50 (2%)	3/49 (6%)	6/50 (12%)	22/50 (44%)	0.0000
Follicular adenoma	4/50 (8%)	16/49 (33%)	36/50 (72%)	36/50 (72%)	0.0000

From Komulainen *et al*. (1997)

Table 6. Aberrant crypt foci (ACF) in the colon of BALB/cA mice after intrarectal administration of MX and intraperitoneal injections of azoxymethane

Treatment (mg/kg bw)	Incidence[a]	ACF/Colon[b]	Crypts/ACF[c]
NaCl + water (control)	8/11	4.0 ± 3.7	1.7 ± 1.5
NaCl + MX (20)	7/11	3.6 ± 4.3	2.6 ± 3.6
NaCl + MX (40)	9/9	3.7 ± 1.9	3.4 ± 1.6[d]
AOM + water	10/11	5.6 ± 4.6	1.9 ± 1.0
AOM + MX (20)	9/11	4.8 ± 4.6	2.3 ± 1.7
AOM + MX (40)	6/6	4.2 ± 3.2	2.2 ± 1.2

From Steffensen *et al*. (1999)
AOM, azoxymethane
[a] Number of animals with ACF/number of animals in the group
[b] Number of ACF/animal (± standard deviation)
[c] Number of aberrant crypts/ACF (± standard deviation)
[d] Statistically significant compared with control value ($p = 0.029$, Student *t*-test)

3.2.2 *Rat*

Groups of 30 male Wistar rats, 6 weeks of age, were given 100 ppm [µg/mL] *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) in the drinking-water for 8 weeks (initiation phase), after which they were given drinking-water containing 0, 10 or 30 ppm [µg/mL] MX (97% pure) for 57 weeks (promotion phase). Three groups of 10 animals received the vehicle control only during the initiation phase and, after 8 weeks, were given 0, 10 or 30 ppm MX in the drinking-water for 57 weeks. Tumour incidence was analysed using the Fisher's or chi-square test. Tumour multiplicities and organ weights were analysed using the Student's *t*-test. No differences in survival were measured for any of the treatment groups when compared with the controls. Four rats in the group that received no initiation before 30 ppm MX died before the termination of the experiment and were not included in the study. Gross and histopathological analyses were determined only for those animals that survived the 57 weeks. Water consumption did not differ among the treatment and control groups (mean water consumption, 21.2–22.7 g per animal per day). Total MX intake for the groups receiving 10 ppm and 30 ppm MX was 0.08 and 0.24 g per animal, respectively. With the exception of the left lung in the MNNG-initiated rats given 30 ppm MX, no significant differences in the final body or selected organ weights were found among the treatment and control groups. MX without prior initiation with MNNG was ineffective in increasing the incidence of cancerous (adenocarcinoma) or precancerous (atypical hyperplasia) lesions in the rat glandular stomach (fundus or pylorus) (Table 7). Other than atypical hyperplasia in 2/9 (30%) rats receiving 30 ppm MX alone, no other lesions were noted. The precancerous and cancerous lesions in the groups treated with

Table 7. Incidence of proliferative lesions in the glandular stomach of rats treated with MNNG and/or MX

Treatment	Adenocarcinoma[a]	Atypical hyperplasia[a]
MNNG/30 µg/mL MX	8/27 (29.6%)[b,c]	25/27 (92.5%)[c]
MNNG/10 µg/mL MX	7/27 (25.9%)	26/27 (96.2%)[d]
MNNG	1/26 (3.8%)	16/26 (61.5%)
30 µg/mL MX	0/9	2/9 (22.2%)
10 µg/mL MX	0/10	0/10
Vehicle control	0/10	0/10

From Nishikawa *et al*. (1999)
MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine
[a] Total lesions in the glandular stomach (fundus and pylorus)
[b] Number of animals with a lesion/number of animals examined
[c] Statistically significant when compared with animals treated with MNNG alone ($p < 0.05$)
[d] Statistically significant when compared with animals treated with MNNG alone ($p < 0.01$)

MNNG were found mainly in the pyloric portion of the glandular stomach. MNNG alone induced atypical hyperplasia in the glandular stomach (16/26, 61.5%). Treating the initiated animals with 10 and 30 ppm MX increased atypical hyperplasia in the glandular stomach to 26/27 (96.2%) and 25/27 (92.5%), respectively. The incidence of adenocarcinomas in the glandular stomach was increased by 30 ppm MX (8/27, 29.6%) compared with MNNG alone (1/26, 3.8%). Cholangiocarcinoma, cholangioma and bile duct hyperplasia were found in the livers of all groups except the drinking-water controls. Treatment with MX enhanced the combined incidence of cholangiocarcinoma and cholangioma: 13% (30 ppm) and 17% (10 ppm MX) when compared with the MNNG control (4%), but the increases were not statistically significant. Thyroid follicular-cell hyperplasia was found only in the groups treated with MNNG and 30 ppm MX (8%) and 10 ppm MX (5%) (Nishikawa *et al.*, 1999).

4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

No data were available for the Working Group.

4.1.2 *Experimental systems*

Pharmacokinetics were evaluated in male Wistar rats after administration of 20 mg/kg of [^{14}C]MX (97% purity) by gavage in deionized water (Komulainen *et al.*, 1992). Blood samples, urine and faeces were collected over 72 h and radioactivity was determined in tissues. Overall, 94% of the administered radioactivity was recovered from the animals after 72 h: 56% in faeces, 35% in urine, 2% in tissues, 1% in blood and 0.1% in the contents of the intestines. Of the radioactivity excreted in urine, 77% appeared within 12 h and 90% within 24 h. At 72 h, the highest concentration of radioactivity in tissues was detected in kidneys, followed by the liver. Also, the level of radioactivity in whole blood was threefold higher than that in serum, suggesting binding of radioactivity to blood cells.

In another experiment (Komulainen *et al.*, 1992), a dose of 2 mg/kg [^{14}C]MX was administered to Wistar rats and radioactivity was determined in tissues after 2 and 6 h. After 2 h, the highest amount of radioactivity was detected in kidneys, followed by the stomach, ileum, urinary bladder and liver. At 6 h, the concentration of radioactivity was markedly decreased in kidneys and urinary bladder and to a lesser extent in the gastrointestinal tract.

After administration of 1 mg/kg [^{14}C]MX intravenously or by gavage to male Wistar rats (Komulainen *et al.*, 1992), elimination of the radioactivity from blood was much slower after intravenous than after oral administration (elimination half-life, 22.9 h versus 3.8 h). The elimination followed bi-phasic kinetics. Approximately 8% of the intravenous dose was detected in faeces, suggesting biliary excretion of MX or its metabolites. In a further study with metabolic cages, no radioactivity was detected in exhaled air ($n = 2$).

Radiolabelled MX (96% pure) was given to male Fischer rats in water by gavage at approximately 40 mg/kg bw (Ringhand *et al.*, 1989). Urine and faecal samples were collected up to 48 h after exposure. Within 48 h, 33.5% of the administered dose was recovered in the urine, mostly within 24 h, 47% in faeces and less than 1% in exhaled air. Some tissues still retained traces of radioactivity after 48 h: 1.6% in muscles, 1.2% in blood, 1.2% in the liver, 0.5% in kidneys and 2.3% in the gastrointestinal contents. None of the radioactivity in urine or faeces was recovered as MX.

Clark and Chipman (1995) studied the intestinal absorption of MX *in vitro* in the everted gut sac system (ileum) of rats. Transport of MX-derived mutagenicity was measured from the mucosal to the serosal side using the standard reverse mutation asssay in *Salmonella typhimurium* TA100. A low, dose-dependent absorption of MX-derived mutagenicity was observed, which became significant at and above 50 μg/mL [230 μM] MX. Preincubation of MX with 1 mM glutathione reduced the number of revertants to below detection levels, and depletion of endogenous glutathione potentiated the mutagenicity, suggesting that mucosal glutathione modulates the absorption and/or mutagenicity of the mutagenic compounds. [The Working Group noted that it is not known whether the mutagenic components are MX or metabolites.]

MX (97% pure) was found to bind bovine serum albumin in phosphate-buffered saline (pH 7.2) *in vitro*. The largest fraction of MX (90%) bound reversibly and was

recovered as intact MX, but a minor part bound tightly to protein. Human plasma bound MX in the same manner as bovine albumin (Haataja *et al.*, 1991).

Upon incubation of rat blood with [^{14}C]MX (2 μg/mL) *in vitro*, 42% of the radioactivity was found in plasma, 26% in erythrocyte cell membranes and 32% bound to haemoglobin (Risto *et al.*, 1993). The binding to erythrocytes may partially explain the long elimination half-life of radioactivity from blood observed after systemic administration (Komulainen *et al.*, 1992).

After a single oral administration of 200 or 300 mg/kg bw MX (98% pure) to male Wistar rats, the fraction of intact MX excreted in the urine within 72 h was 0.03–0.07% of the administered dose, and 90% of that appeared within 24 h (n = 3). The metabolites were not identified (Komulainen *et al.*, 1994).

Meier *et al.* (1996) measured MX-derived mutagenicity (Ames test with the TA100 strain) in the urine of male and female Fisher 344 rats administered MX at doses of 0, 8, 16, 32 and 64 mg/kg bw by gavage daily for 14 days. Only urine from the highest-dose group showed significant mutagenic activity, which corresponded to approximately 0.3% of the dose administered daily. Treatment of the urine samples with β-glucuronidase had no effect on the mutagenicity of any samples. The result was interpreted to indicate nearly complete metabolism of MX. [The Working Group noted that it is not known whether the mutagenic components are MX or metabolites.]

4.2 Toxic effects

4.2.1 *Humans*

No data were available to the Working Group.

4.2.2 *Experimental systems*

MX (> 99% pure) in water was administered by gavage to male and female Swiss-Webster mice at doses of 10, 20, 42, 88 or 184 mg/kg bw on 2 consecutive days and the animals were observed for 2 weeks (Meier *et al.*, 1987a). All animals given the highest dose died, and 9/10 died within 24 h. At necropsy, enlarged stomach and moderate haemorrhaging in the forestomach were observed. Doses of 88 mg/kg bw or less did not cause death or any clinical signs of toxicity. The acute 50% lethal dose (LD_{50}) was estimated to be 128 mg/kg bw.

MX (98% pure) in deionized water was administered orally to groups of 10 male Wistar rats at doses of 200, 300, 400 or 600 mg/kg bw to evaluate acute toxicity (Komulainen *et al.*, 1994). The LD_{50} in 48 h was 230 mg/kg bw. Clinical signs included laborious breathing, wheezing, gasping and dyspnoea and decreased motor activity leading to catalepsia and cyanosis before death. At autopsy, the lungs appeared oedematous and spongy. Histological examination showed a strong irritation and necrosis of the mucosa of the entire gastrointestinal tract, and expansion of the stomach. Tubular damage was observed in both kidneys in one animal, characterized by a thin epithelium and dilated tubules.

To evaluate subchronic toxicity in rats, MX (98% pure) in deionized water was administered to groups of 15 male and 15 female Wistar rats on 5 days per week by gavage (Vaittinen *et al.*, 1995). For the low-dose regimen, the dose of 30 mg/kg bw was administered for 18 weeks. For the high-dose regimen, the dose was gradually increased from 45 mg/kg bw for 7 weeks to 60 mg/kg bw for the next 2 weeks to 75 mg/kg bw for the last 5 weeks. Urine and blood samples were collected and full histopathology was performed. The low-dose regimen did not result in any signs of toxicity. At the high-dose regimen, two males and one female died. Food consumption and body weights were significantly decreased in males. Sodium concentration in serum decreased, urine excretion increased and its specific gravity decreased in both sexes, while water consumption increased only in males. Serum cholesterol and triglycerides, as well as the relative weight of the liver and kidneys, increased in a dose-dependent manner in both sexes. Mucosal hyperplasia of the duodenum occurred at both dose levels in both sexes (57% of animals in the high-dose regimen group), accompanied by focal epithelial hyperplasia of the forestomach in males (controls, 1/15; low-dose regimen, 2/15; and high-dose regimen, 3/14) and superficial haemorrhagic necrosis in gastric glandular epithelium in 2/14 females at the high-dose regimen. However, these hyperplasias were thought to result from the local irritating effect of the gavage dosing of MX. Epithelial atypia in the bladder was observed in 1/14 male and 1/14 female in the high-dose group. The target organs of toxicity were the kidneys and the liver.

In the same animals, activities of the xenobiotic metabolizing enzymes 7-ethoxyresorufin-*O*-deethylase (EROD), pentoxyresorufin-*O*-dealkylase (PROD), NADPH-cytochrome-*c*-reductase, UDP-glucuronosyltransferase (UDPGT) and glutathione *S*-transferase (GST) were measured in microsomal and cytosolic fractions of the liver, kidneys, duodenum and lung (Heiskanen *et al.*, 1995). Most changes were observed in the kidneys. MX decreased the activity of the phase I metabolism enzymes EROD and PROD and induced the activity of the phase II conjugation enzymes UDPGT and GST in both sexes. In addition, EROD and GST activities in the liver were similarly affected in both sexes.

In 14-day range-finding studies of a subchronic toxicity study (Vaittinen *et al.*, 1995), male Han-Wistar rats were given MX (> 98% pure) in water by gavage at doses of 12.5, 25, 50, 100 or 200 mg/kg bw daily or at doses of 5, 10 or 20 mg/kg bw on 5 days per week. Daily doses of 100 and 200 mg/kg were lethal to all animals within 5 days and 3/5 animals given 50 mg/kg died within 9 days. Breathing difficulties, nostril discharge and a foamy liquid in the respiratory tract were observed with doses of 25 mg/kg and above. Doses of 5, 10 and 20 mg/kg bw did not cause general toxicity detectable clinically or by macroscopic pathology, but several biochemical changes in serum and urine were observed, particularly in females. Urea and creatinine in serum were increased at all doses and bilirubin at the two highest doses. Inorganic phosphate and potassium were decreased at the two highest doses and chloride concentration at the highest dose only. The pH of urine decreased and the specific gravity increased at the two highest doses in both sexes. The changes in electrolyte balance and urine composition, and the increase in blood urea and

creatinine suggested an acute or subacute effect of MX on kidney function in females. However, no notable changes were observed by histopathology.

Male and female Fischer 344 rats received MX (> 99% pure) by gavage in distilled water at doses of 8, 16, 32 or 64 mg/kg bw for 14 consecutive days (Daniel *et al.*, 1994). At the highest dose in both sexes, body weight gain and food and water consumption decreased, and the relative liver and kidney weight increased significantly. In addition, the relative weight of the testes and thymus in males and of the spleen in females was increased, all at the highest dose only. Serum cholesterol and calcium concentrations increased in a dose-dependent manner in both sexes. In females, blood platelets increased in a dose-dependent fashion and the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were significantly decreased at most doses. In males, the number of erythrocytes and platelets, the concentration of haemoglobin, haematocrit and creatinine, and ALP activity were decreased at the highest dose. Lesions in the forestomach, characterized by epithelial hyperplasia, chronic-active inflammation, hyperkeratosis or ulceration, were the most notable histopathological changes, and were observed in both sexes in 40% of the animals in the highest-dose group. Extramedullary haematopoiesis was slightly more common among MX-treated animals. The liver was considered to be the target organ of toxicity. [The Working Group noted that kidney function was not evaluated thoroughly in this study.]

Administration of MX to male Fischer 344 rats by gavage at a dose of 64 mg/kg bw per day for 4 days caused acute toxicity characterized by pyloric blockage, dilatation of the intestine and nose bleeding. No consistent effect on the peroxisome proliferation enzymes was observed in the liver (Meier *et al.*, 1996). The activity of catalase decreased, that of urate oxidase increased and that of fatty acyl coenzyme A oxidase remained unchanged. The enzymes of glutathione metabolism (γ-glutamylcysteine synthetase [glutamate-cysteine ligase], GST and glutathione peroxidase) were not affected. The phase I metabolism enzymes NADPH-cytochrome-P450 reductase, aminopyrine *N*-demethylase and aryl hydrocarbon hydroxylase were all significantly inhibited. Concomitantly, urinary excretion of thioethers and D-glucaric acid were largely reduced, indicating toxic metabolic effects in the liver.

Daniel *et al.* (1994) administered MX (> 99% pure) to female and male B6C3F_1 mice by gavage at 8, 16, 32 or 64 mg/kg bw for 14 consecutive days with the same study design as for rats (see above). Because the animals were group-housed, a large number of males in all groups displayed extensive skin lesions and chronic inflammation due to fighting. No changes in food or water consumption were observed in any group. MX caused epithelial hyperplasia of the forestomach at all treatment levels in both sexes (20–60% of animals), and extramedullary haematopoiesis in the spleen of females. Extramedullary haematopoiesis was common in control males. In males, body weight gain, spleen weight, total white blood cell count, the distribution of neutrophils, ALT activity and creatinine concentrations were significantly decreased; distribution of lymphocytes and eosinophils, ALP activity in serum and the relative weight of the thymus, testes and adrenal glands were increased in all treatment groups, generally in a dose-dependent manner. In the highest-

dose group only, AST, lactate dehydrogenase and blood urea nitrogen were also decreased in serum. In females, the changes showing a clear dose relation were an increase in neutrophils (except at the highest dose) and a decrease in lymphocytes and haematocrit, ALP activity and thymus weight. At the highest dose only, the number of erythrocytes, the concentration of haemoglobin and blood urea nitrogen, and the weight of adrenal glands had decreased in females. Because of inconsistency in changes, no clear target organ of toxicity could be defined in mice in this study. [The Working Group noted that chronic inflammation and stress were likely to have contributed to the results observed in males.]

After a single dose of 0, 10, 30 or 60 mg/kg bw by gavage in 0.02 M HCl, MX [purity not specified] stimulated DNA single-strand scissions, replicative DNA synthesis and ornithine decarboxylase activity in the pyloric mucosa of the stomach in male Fischer 344 rats. The effect was dose-dependent and indicated an increase in cell proliferation (Furihata *et al*., 1992).

Male Wistar rats were administered MX (97% pure) at concentrations of 6.25, 12.5, 25 and 50 μg/mL in the drinking-water for 5 weeks. Doses of 12.5 μg/mL and above stimulated cell proliferation (a twofold increase) in the mucosal epithelium of the glandular stomach but not in the pyloric mucosa. This was accompanied by focal gastric erosion at the two highest doses. Lipid peroxidation in the gastric mucosa was marginally increased in a loose dose-dependent manner, and urine volume and urinary lipid peroxidation products were significantly increased at 12.5 and 25 μg/mL (Nishikawa *et al.*, 1994).

In male and female Wistar rats given 0, 1, 10 or 60 mg/kg bw (40 mg/kg for females) for 7 or 21 days, MX did not cause any morphological changes in thyroid glands, adrenal glands or liver after either time-point. No notable changes were seen in staining for proliferating cell nuclear antigen between control and treated animals in these organs (Komulainen *et al.*, 2000a).

MX (> 99% pure) slightly decreased glutathione levels at 100 μM and above in human white blood cells *in vitro*, but no change was observed in the level of free intracellular calcium (Nunn & Chipman, 1994).

4.3 Reproductive, developmental and hormonal effects

4.3.1 *Humans*

No data were available to the Working Group.

4.3.2 *Experimental systems*

MX showed a dose-dependent decrease in differentiation of foci in the micromass test system *in vitro* in rat embryo midbrain cells and limb bud cells exposed to concentrations of 1, 2, 5 or 10 μg/mL [4.6, 9.2, 23 and 46 μM, respectively], in the absence of metabolic activation. Concomitantly, MX inhibited differentiation of both cell types with a 50% inhibition concentration (IC_{50}) of about 3 μg/mL [13 μM]. Metabolic activation eliminated the effect (Teramoto *et al*., 1998). Preincubation of MX in tissue culture medium, which is

known to degrade MX, also restored differentiation of foci, supporting the concept that degradation products of MX were not the responsible components (Teramoto *et al.*, 1999).

To evaluate the effects of MX on blood levels of thyroid-stimulating hormone (TSH), thyroxine (T_4), triiodothyronine (T_3), prolactin and growth hormone, groups of male and females Wistar rats were administered MX (> 97% pure) by gavage in deionized water at levels of 0, 1, 10 or 60 mg/kg bw (or 40 mg/kg for females) daily for 7 or 21 days (Komulainen *et al.*, 2000a). MX did not affect blood levels of TSH, T_4 or prolactin. In males, levels of T_3 increased transiently at the highest dose, whereas those of growth hormone decreased transiently at all doses. MX did not affect the levels of TSH, prolactin or growth hormone in blood 2 h after a single dose. It was concluded that MX does not cause dysregulation of the thyroid–pituitary axis and that the tumorigenicity of MX to the thyroid follicular epithelium in rats does not result from hormonal imbalance.

Blood concentrations of TSH, T_4 and T_3 were also measured in male and female Wistar rats in a 104-week carcinogenicity study (Komulainen *et al.*, 1997; see Section 3.1.2) in all animals surviving to the end of the study. No significant differences in hormone levels were observed at any dose compared with control groups.

4.4 Genetic and related effects

4.4.1 *Humans*

No data were available to the Working Group.

4.4.2 *Experimental systems* (see Table 8 for details and references)

MX was highly mutagenic to all but one *Salmonella typhimurium* strain tested, and one of the most potent mutagens in the TA100 strain ever tested. MX was also mutagenic in most strains of *Eschericia coli* tested. MX acts as a direct-acting mutagen, the activity of which was largely decreased or eliminated by metabolic activation. MX caused DNA damage (including unscheduled DNA synthesis) in purified DNA and in all animal and human cells studied *in vitro* except in rat hepatocytes in one study. Inhibitors of DNA repair enzymes potentiated the effect of MX in rapidly dividing cells by a factor of 10–100. MX caused gene mutations, sister chromatid exchange and chromosomal aberrations in all animal and human cells studied *in vitro*. Also, it caused apoptosis in human HL-60 cells (Marsteinstredet *et al.*, 1997b). MX was positive in a two-stage cell transformation assay in CH3 10T1/2 cells as either an initiator or promoter. A metabolic cooperation assay in Chinese hamster V79 cells showed a tentative inhibition of intercellular communication *in vitro*. Glutathione, L-cysteine, several other nucleophiles, tissue culture medium and serum strongly decreased or eliminated the mutagenic and genotoxic effects of MX *in vitro* (Ishiguro *et al.*, 1987; Mäki-Paakkanen *et al.*, 1994; Matsumura *et al.*, 1994; Watanabe *et al.*, 1994). MX reacted directly with glutathione, and GST catalysed the reaction (Meier *et al.*, 1990), leading to a further inhibition of MX-induced mutagenicity. Partial depletion

Table 8. Genetic and related effects of MX

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
DNA strand breaks and apurinic/apyrimidinic sites, PM2 DNA, *in vitro*	+	NT	[217 µg/mL]	Hyttinen & Jansson (1995)
DNA damage, cleavage of ΦX174, *in vitro*	+	NT	0.92 µg/mL[c]	LaLonde & Ramdayal (1997)
DNA damage, *Escherichia coli* PQ37, SOS chromotest	+	(–)	0.4–3.3 ng/mL	Tikkanen & Kronberg (1990)
Differential DNA repair, *Escherichia coli* K-12 343/113	+	(+)	4 ng/mL	Fekadu *et al.* (1994)
Prophage-induction assay, *Escherichia coli* $WP2_s$ (λ)	+	+	10.9 ng/mL	DeMarini *et al.* (1995)
Salmonella typhimurium TM677, forward mutation for 8-azaguanine resistance	+	NT	10.9 ng/mL	DeMarini *et al*. (1995)
Salmonella typhimurium TA100, reverse mutation	+	(+)	29 rev/ng	Ishiguro *et al*. (1987)
Salmonella typhimurium TA100, reverse mutation	+	+	64 rev/ng	Meier *et al.* (1987b)
Salmonella typhimurium TA100, reverse mutation	+	–	29 rev/ng	Tikkanen & Kronberg (1990)
Salmonella typhimurium TA100, reverse mutation	+	NT	18 rev/ng	LaLonde *et al*. (1991a)
Salmonella typhimurium TA100, reverse mutation	+	NT	14 rev/ng	LaLonde *et al*. (1991b)
Salmonella typhimurium TA100, reverse mutation	+	NT	22 rev/ng	DeMarini *et al*. (1995)
Salmonella typhimurium TA100, reverse mutation	+	NT	20 rev/ng	Hyttinen *et al.* (1995)
Salmonella typhimurium TA100, reverse mutation	+	NT	18 rev/ng	Jansson *et al*. (1995)
Salmonella typhimurium TA100, reverse mutation	+	NT	19 rev/ng	Knasmüller *et al*. (1996)
Salmonella typhimurium TA100, reverse mutation	+	NT	18 rev/ng	LaLonde *et al*. (1997)
Salmonella typhimurium TA100, reverse mutation	+	NT	38 rev/ng	Yamada *et al.* (1997)
Salmonella typhimurium TA100, reverse mutation	+	NT	54 rev/ng	Franzén *et al.* (1998a)

Table 8 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA100, reverse mutation	+	–	4.5 rev/ng	Kargalioglu *et al*. (2002)
Salmonella typhimurium TA102, reverse mutation	+	NT	6.35 rev/ng	Meier *et al.* (1987b)
Salmonella typhimurium TA104, reverse mutation	+	NT	39 rev/ng	Franzén *et al.* (1998a)
Salmonella typhimurium TA1535, reverse mutation	+	NT	0.48 rev/ng	Meier *et al.* (1987b)
Salmonella typhimurium TA1535, reverse mutation	(+)	NT	0.04 rev/ng	DeMarini *et al*. (1995)
Salmonella typhimurium TA1535, reverse mutation	+	NT	0.1 rev/ng	Jansson *et al*. (1995)
Salmonella typhimurium TA1538, reverse mutation	+	NT	0.13 rev/ng	Meier *et al.* (1987b)
Salmonella typhimurium TA98, reverse mutation	+	NT	9.18 rev/ng	Meier *et al.* (1987b)
Salmonella typhimurium TA98, reverse mutation	+	–	0.89 rev/ng	Tikkanen & Kronberg (1990)
Salmonella typhimurium TA98, reverse mutation	+	NT	2.60 rev/ng	DeMarini *et al*. (1995)
Salmonella typhimurium TA98, reverse mutation	+	NT	6.1 rev/ng	Franzén *et al.* (1998a)
Salmonella typhimurium TA98, reverse mutation	+	+	0.53 rev/ng	Kargalioglu *et al*. (2002)
Salmonella typhimurium TA92, reverse mutation	+	NT	0.32 rev/ng	Meier *et al.* (1987b)
Salmonella typhimurium TA97, reverse mutation	+	NT	7.26 rev/ng	Meier *et al.* (1987b)
Salmonella typhimurium TA97, reverse mutation	+	–	4.8 rev/ng	Tikkanen & Kronberg (1990)
Salmonella typhimurium UTH8414, reverse mutation	+	NT	0.20 rev/ng	DeMarini *et al*. (1995)
Salmonella typhimurium UTH8413, reverse mutation	+	NT	0.08 rev/ng	DeMarini *et al*. (1995)
Salmonella typhimurium TP2428, reverse mutation	+	NT	0.4 rev/ng	Knasmüller *et al*. (1996)
Salmonella typhimurium TA1950, reverse mutation	+	NT	0.04 rev/ng	Knasmüller *et al*. (1996)

Table 8 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium YG7112, reverse mutation	+	NT	26.4 rev/ng	Yamada *et al*. (1997)
Salmonella typhimurium YG7113, reverse mutation	+	NT	26.2 rev/ng	Yamada *et al*. (1997)
Salmonella typhimurium YG7119, reverse mutation	+	NT	33 rev/ng	Yamada *et al*. (1997)
Salmonella typhimurium RSJ100, reverse mutation	–	–	1.63 µg/mL	Kargalioglu *et al*. (2002)
Escherichia coli CC102, CC104, reverse mutation	–	NT	20 µg/plate	Watanabe-Akanuma & Ohta (1994)
Escherichia coli CC107, CC109, CC111, reverse mutation	+	NT	5 µg/plate	Watanabe-Akanuma & Ohta (1994)
Escherichia coli CC108, CC110, reverse mutation	+	NT	2 µg/plate	Watanabe-Akanuma & Ohta (1994)
Escherichia coli ZA2102, ZA2104, reverse mutation	+	NT	0.05 µg/plate	Watanabe-Akanuma & Ohta (1994)
Escherichia coli ZA2108, ZA2109, ZA2110, reverse mutation	+	NT	0.1 µg/plate	Watanabe-Akanuma & Ohta (1994)
Escherichia coli ZA2107, ZA2111, reverse mutation	+	NT	0.2 µg/plate	Watanabe-Akanuma & Ohta (1994)
Escherichia coli ZA4102, ZA4104, reverse mutation	–	NT	20 µg/plate	Watanabe-Akanuma & Ohta (1994)
Escherichia coli ZA4107, ZA4108, ZA4109, ZA4110, ZA4111, reverse mutation	+	NT	5 µg/plate	Watanabe-Akanuma & Ohta (1994)
Eschericia coli ZA5102, reversion mutation	+	NT	0.1 µg/plate	Watanabe-Akanuma & Ohta (1994)
Eschericia coli ZA5104, ZA5108, ZA5109, reversion mutation	+	NT	0.2 µg/plate	Watanabe-Akanuma & Ohta (1994)

Table 8 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Eschericia coli ZA5107, ZA5110, ZA5111, reverse mutation	–	NT	1 µg/plate	Watanabe-Akanuma & Ohta (1994)
DNA damage, rat hepatocytes, rat testicular cells, Chinese hamster V79 cells *in vitro* (alkaline elution)	+	NT	6.5 µg/mL	Brunborg *et al*. (1991)
DNA strand breaks, rat hepatocytes *in vitro* (DNA alkaline unwinding assay)	–	NT	11 µg/mL	Chang *et al*. (1991)
DNA single-strand breaks or alkali-labile sites, pig LLC-PK_1 cells *in vitro* (without pretreatment with DNA repair enzyme inhibitors, alkaline elution)	+	NT	65 µg/mL	Holme *et al*. (1999)
DNA single-strand breaks or alkali-labile sites, pig LLC-PK_1 cells *in vitro* (with pretreatment with DNA repair enzyme inhibitors, alkaline elution)	+	NT	6.5 µg/mL	Holme *et al*. (1999)
DNA single-strand breaks or alkali-labile sites, rat testicular cells *in vitro* (with and without pretreatment with DNA repair enzyme inhibitors, alkaline elution)	+	NT	22 µg/mL	Holme *et al*. (1999)
DNA strand breaks and alkali-labile damage, Chinese hamster ovary cells *in vitro* (single-cell gel electrophoresis assay)	+	NT	8 µg/mL	Mäki-Paakkanen *et al.* (2001)
Unscheduled DNA synthesis, male Wistar rat and BALB/c mouse hepatocytes *in vitro*	+	NT	0.43 µg/mL	Nunn *et al*. (1997)
Unscheduled DNA synthesis, rat hepatocytes *in vitro*	+	NT	3.6 µg/mL	Le Curieux *et al*. (1999)
Gene mutation, Chinese hamster ovary cells, *Hprt* locus *in vitro*	+	NT	2.5 µg/mL	Jansson & Hyttinen (1994)
Gene mutation, Chinese hamster ovary cells, ouabain resistance *in vitro*	+	NT	2 µg/mL	Mäki-Paakkanen *et al.* (1994)
Gene mutation, Chinese hamster V79 cells, *Hprt* locus *in vitro*	–	NT	1.1 µg/mL	Brunborg *et al*. (1991)
Gene mutation, Chinese hamster V79 cells, 6-TG resistance *in vitro*	+	NT	1 µg/mL	Matsumura *et al.* (1994)
Gene mutation, mouse lymphoma L5178Y cells, *Tk* locus *in vitro*	+	NT	0.5 µg/mL	Harrington-Brock *et al*. (1995)

Table 8 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Sister chromatid exchange, Chinese hamster V79 cells *in vitro*	+	NT	0.43 µg/mL	Brunborg *et al*. (1991)
Sister chromatid exchange, Chinese hamster ovary cells *in vitro*	+	NT	0.19 µg/mL	Mäki-Paakkanen *et al.* (1994)
Sister chromatid exchange, Chinese hamster ovary cells *in vitro*	+	NT	0.24 µg/mL	Mäki-Paakkanen *et al.* (2001)
Sister chromatid exchange, rat peripheral lymphocytes *in vitro*	+	NT	20 µg/mL	Jansson *et al*. (1993)
Micronucleus formation, mouse lymphoma L5178Y cells *in vitro*	+	NT	11 µg/mL	Le Curieux *et al*. (1999)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro*	+	+	4 µg/mL	Meier *et al.* (1987b)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro*	+	NT	4 µg/mL	Mäki-Paakkanen *et al.* (1994)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro*	+	NT	0.5 µg/mL	Mäki-Paakkanen *et al.* (2001)
Chromosomal aberrations, mouse lymphoma L5178Y cells *in vitro*	+	NT	0.75 µg/mL	Harrington-Brock *et al*. (1995)
Chromosomal aberrations, rat peripheral lymphocytes *in vitro*	+	NT	60 µg/mL	Jansson *et al*. (1993)
Cell transformation, C3H 10T1/2 mouse cells *in vitro*	+	NT	10 µg/mL	Laaksonen *et al.* (2001)
DNA strand breaks, human lymphoblastoid cell line CCRF-CEM *in vitro* (DNA alkaline unwinding assay)	+	+	9.6 µg/mL	Chang *et al*. (1991)
DNA strand breaks, human white blood cells *in vitro* (DNA alkaline unwinding assay)	+	NT	0.22 µg/mL	Nunn & Chipman (1994)
DNA strand breaks or alkali-labile sites, HL-60 cells *in vitro* (without pretreatment with DNA repair enzyme inhibitors, alkaline elution)	+	NT	21.7 µg/mL	Marsteinstredet *et al*. (1997a)

Table 8 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
DNA strand breaks or alkali-labile sites, HL-60 cells *in vitro* (with pretreatment with DNA repair enzyme inhibitors, alkaline elution)	+	NT	0.22 µg/mL	Marsteinstredet *et al*. (1997a)
DNA strand breaks or alkali-labile sites, resting human leukocytes *in vitro* (with and without pretreatment with DNA repair inhibitors, alkaline elution)	+	NT	21.7 µg/mL	Holme *et al*. (1999)
DNA strand breaks or alkali-labile sites, proliferating human leukocytes *in vitro* (without pretreatment with DNA repair enzyme inhibitors, alkaline elution)	+	NT	21.7 µg/mL	Holme *et al*. (1999)
DNA strand breaks or alkali-labile sites, proliferating human leukocytes *in vitro* (with pretreatment with DNA repair enzyme inhibitors, alkaline elution)	+	NT	0.65 µg/mL	Holme *et al*. (1999)
DNA strand breaks or alkali-labile sites, HL-60 cells *in vitro* (without pretreatment with DNA repair enzyme inhibitors, alkaline elution)	+	NT	65 µg/mL	Holme *et al*. (1999)
DNA strand breaks or alkali-labile sites, HL-60 cells *in vitro* (with pretreatment with DNA repair enzyme inhibitors, alkaline elution)	+	NT	0.65 µg/mL	Holme *et al*. (1999)
Unscheduled DNA synthesis, human hepatocytes *in vitro*	+	NT	0.43 µg/mL	Nunn *et al.* (1997)
Gene mutation, human B-lymphoblastoid cell lines MCL-5, AHH-1 $TK^{+/-}$, h1A1v2, *TK* locus *in vitro*	+	NT	3 µg/mL	Woodruff *et al.* (2001)
DNA damage, male Wistar rat liver, kidney, lung, testis, stomach, duodenum, colon, urinary bladder, bone marrow cells *in vivo* (alkaline elution)	–		125 po, 1 h	Brunborg *et al.* (1991)
DNA single-strand breaks, male Fischer 344 rat pyloric mucosa of stomach *in vivo* (alkaline elution)	+		48 po, 2 h	Furihata *et al.* (1992)

Table 8 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
DNA strand breaks and alkali-labile damage, male CD-1 mouse liver, kidney, lung, brain, stomach, jejunum, ileum, colon, bladder cells *in vivo* (single cell gel electrophoresis assay)	+		100 po, 1, 3, 6, 24 h	Sasaki *et al.* (1997)
DNA strand breaks and alkali-labile damage, male CD-1 mouse spleen, bone-marrow cells *in vivo* (single-cell gel electrophoresis assay)	–		100 po, 1, 3, 6, 24 h	Sasaki *et al.* (1997)
DNA damage, male B6C3F_1 mouse liver, kidney, spleen, colon cells *in vivo* (with pretreatment with DNA repair enzyme inhibitors, alkaline elution)	–		80 ip, 1 h	Holme *et al.* (1999)
DNA damage, male B6C3F_1 mouse liver, kidney cells *in vivo* (with pretreatment with DNA repair enzyme inhibitors, alkaline elution)	+		40 ip, 1 h	Holme *et al.* (1999)
DNA damage, male B6C3F_1 mouse spleen, colon cells *in vivo* (with pretreatment with DNA repair enzyme inhibitors, alkaline elution)	–		80 ip, 1 h	Holme *et al.* (1999)
Differential DNA repair, male Swiss albino mouse liver, lung, kidney, spleen, stomach, intestines *in vivo*, measured in two strains of *E. coli* K-12 indicator cells recovered from organs	+		4.3 po, 2 h	Fekadu *et al.* (1994)
Unscheduled DNA synthesis, BALB/c hepatocytes *ex vivo*	–		100 po, 16 h	Nunn *et al.* (1997)
Sister chromatid exchange, Wistar rat peripheral lymphocytes *in vivo*	+		30 po, 18 weeks	Jansson *et al.* (1993)
Sister chromatid exchange, male Wistar rat kidney *in vivo*	+		25, po × 3	Mäki-Paakkanen & Jansson (1995)
Sister chromatid exchange, male Wistar rat peripheral lymphocytes *in vivo*	+		100, po × 3	Mäki-Paakkanen & Jansson (1995)
Micronucleus formation, Swiss-Webster mouse, bone-marrow polychromatic erythrocytes *in vivo*	–		90 po × 2	Meier *et al.* (1987b)
Micronucleus formation, NMRI mouse bone-marrow polychromatic erythrocytes *in vivo*	–		8.8 ip	Tikkanen & Kronberg (1990)

Table 8 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Nuclear anomalies (including micronuclei), male B6C3F$_1$ mouse forestomach, duodenum *in vivo*	+		80 po	Daniel *et al.* (1991)
Nuclear anomalies (including micronuclei), male B6C3F$_1$ mouse colon *in vivo*	–		100 po	Daniel *et al.* (1991)
Micronucleus formation, B6C3F$_1$ mouse blood polychromatic erythrocytes *in vivo*	–		64 po, 14 days	Meier *et al.* (1996)
Micronucleus formation, male Wistar rat peripheral blood lymphocytes *in vivo*	+		100 po × 3	Mäki-Paakkanen & Jansson (1995)
Micronucleus formation, Wistar rat bone-marrow polychromatic erythrocytes *in vivo*	–		approx. 5, 104 weeks	Jansson (1998)
Inhibition of intercellular communication, Chinese hamster V79 cells *in vitro* (metabolic cooperation assay)	(+)	NT	0.8 µg/mL	Matsumura *et al.* (1994)

[a] +, positive; (+), weak positive; –, negative; NT, not tested

[b] LED, lowest effective dose; HID, highest ineffective dose; in-vitro tests, as indicated; in-vivo tests, mg/kg bw per day; po, oral; ip, intraperitoneal; rev, revertant

[c] Only dose tested

of glutathione in cells had little effect on MX-induced DNA damage (Brunborg *et al.*, 1991; Chang *et al.*, 1991).

The *cis* arrangement of the $CHCl_2$ (position 4) and Cl (position 3) substituents in MX is essential for the high mutagenicity in *S. typhimurium* TA100 (Ishiguro *et al*., 1987, 1988; LaLonde *et al.,* 1991c). The OH group at position 5 and Cl at position 3 and 6 are also important for the mutagenic potency (LaLonde *et al*., 1991b,c).

Results on DNA damage *in vivo* are less clear. A single oral dose of MX to mice caused DNA damage in a number of tissues, but not in spleen or bone marrow. Following intraperitoneal administration at a lower dose and with a shorter follow-up period, DNA damage was observed only after pretreatment of the animals with DNA-repair enzyme inhibitors, and only in the liver and kidney. MX-induced DNA damage was repaired rapidly (within 3 h) in mouse liver but more slowly in other tissues (Sasaki *et al.*, 1997). In rats, MX did not induce DNA damage in any of the tissues examined, except in the pyloric mucosa of the stomach following oral administration in a single study. Oral administration of MX to mice caused DNA damage in *E. coli* K-12 indicator cells recovered from a number of tissues.

In rats, MX caused sister chromatid exchange in peripheral blood lymphocytes in two studies and in kidney cells in a single study *in vivo* after repeated doses, but had no effect on micronucleus formation in bone marrow or blood polychromatic erythrocytes in any of four studies. Micronuclei were observed in rat peripheral blood lymphocytes in a single study, and nuclear anomalies (including micronuclei) in mouse forestomach and duodenum in a single study, both after oral administration of MX.

The predominant base-pair substitution induced by MX is a GC→TA transversion, observed in several *S. typhimurium* strains at the *hisG* locus (DeMarini *et al.*, 1995; Hyttinen *et al.*, 1995; Knasmüller *et al*., 1996; Shaughnessy *et al.*, 2000) and in Chinese hamster ovary cells at the *Hprt*-locus (Hyttinen *et al.*, 1996). In *S. typhimurium* TA100, the hot spot was in the second position of the *hisG46* target CCC codon. MX also caused duplications, frameshift mutations of a two-base deletion and frameshifts with adjacent base substitution in *S. typhimurium* TA98 (DeMarini *et al.*, 1995), and frameshift mutations of one- or two-base insertion or deletion in several strains of *E. coli* (Watanabe-Akanuma & Ohta, 1994). AT→TA transversions, deletions of single base pairs, larger deletions and insertions in cDNA were reported in Chinese hamster ovary cells (Hyttinen *et al*., 1996).

MX produced DNA adducts *in vitro* under various reaction conditions but not in all experiments (Alhonen-Raatesalmi & Hemminki, 1991). Five different adducts were reported to be formed in buffered solutions with MX *in vitro*, three with 2′-deoxyadenosine (Le Curieux *et al.*, 1997; Munter *et al.*, 1998) and two with guanosine (Franzén *et al*., 1998b; Munter *et al*., 1999). The guanosine adduct described by Franzén *et al*. (1998b) could not be confirmed in the later study (Munter *et al*., 1999). Two of the adducts formed with 2′-deoxyadenosine were also observed in calf thymus DNA reacted with MX *in vitro* (Le Curieux *et al.*, 1997; Munter *et al.*, 1998) but not the guanosine adduct (Munter *et al*., 1999). All adducts were observed after incubation for several days.

Altogether four point mutations were found in *p53* (exon 4–7) in 47 different MX-induced liver tumours in Wistar rats (Komulainen *et al*., 1997, 2000b). No consistent pattern of point mutations was observed. Exons 1 and 2 of Ki-*ras*, Ha-*ras* and N-*ras* of the same tumours did not contain any mutations. MX did not alter the expression of p53 protein in the normal liver (Komulainen *et al.*, 2000b) or in thyroid gland tumours of these animals (Hakulinen *et al.*, 2002). The expression of p21 Ki-ras protein was also unaffected in MX-induced thyroid gland tumours (Hakulinen *et al.*, 2002).

4.5 Mechanistic considerations

In-vitro data in bacteria and in mammalian cells indicate that MX is genotoxic. In addition, it causes DNA damage *in vivo*. Data on mutagenicity tests *in vitro*, mutation spectra and adduct formation suggest that the guanine moiety may be one target of MX in DNA. In addition, MX was 100-fold more mutagenic in the form of lactone (closed-ring form) than in the open-ring conformation *in vitro* (LaLonde *et al.,* 1991b), suggesting that the closed-ring conformation may be responsible for the mutagenicity at physiological pH. However, examination of the structural and electronic properties of MX have not yet identified the form of interaction of MX with DNA.

Studies on hormonal effects of MX suggest that it does not cause thyroid gland tumours in rats by the TSH-mediated promotion mechanism (Komulainen *et al.*, 1997, 2000a).

5. Summary of Data Reported and Evaluation

5.1 Exposure data

3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) is a disinfection by-product that has been found at nanogram-per-litre levels in drinking-water as a result of chlorination or chloramination.

5.2 Human carcinogenicity data

Several studies were identified that analysed risk with respect to one or more measures of exposure to complex mixtures of disinfection by-products that are found in most chlorinated and chloraminated drinking-water. No data specifically on MX were available to the Working Group.

5.3 Animal carcinogenicity data

In a single bioassay, MX induced malignant and benign thyroid and mammary tumours in male and female rats. MX failed to increase the incidence of tumours in the small intestine or colon of C57BL/6J-MiN$^{-/-}$ mice. MX promoted preneoplastic and malignant lesions

in the glandular stomach but not aberrant crypt foci (preneoplastic lesions) in the colon of rats initiated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.

5.4 Other relevant data

MX is well absorbed in the gastrointestinal tract of experimental animals. About 40% of the administered dose is excreted rapidly in urine almost entirely as uncharacterized metabolites. Pharmacokinetic studies suggest that MX does not accumulate upon continuous exposure.

The target organs of toxicity of MX in rats are the kidneys and the liver. It affects hepatic lipid metabolism and kidney function. At high doses, MX is highly irritating in the gastrointestinal tract. No data were available on the teratogenicity of MX *in vivo*.

MX is genotoxic. It is a direct-acting mutagen and causes DNA and chromosome damage *in vitro* and DNA damage *in vivo*. MX-induced liver tumours of rats contained only a few point mutations in *p53* and none in *ras* genes. Hormonal data suggest that MX does not cause thyroid gland tumours in rats by the thyroid-stimulating hormone-mediated promotion mechanism.

5.5 Evaluation

There is *inadequate evidence* in humans for the carcinogenicity of MX.

There is *limited evidence* in experimental animals for the carcinogenicity of MX.

MX is a potent, direct-acting mutagen that induces primarily GC→TA transversions in both bacterial and mammalian cells. It induces DNA damage in bacterial and mammalian cells as well as in rodents *in vivo*. MX is a chromosomal mutagen in mammalian cells and in rats, and it induces mammalian cell transformation *in vitro*. The MX-associated thyroid gland tumours in rats are caused by mechanisms other than TSH-mediated hormonal promotion.

Overall evaluation

3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) is *possibly carcinogenic to humans (Group 2B)*.

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