EVIDENCE ON THE CARCINOGENICITY OF

MX

(3-chloro-4-(dichloromethyl)-5-hydroxy-2(*5H*)-furanone)

DRAFT

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Reproductive and Cancer Hazard Assessment Section
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PREFACE

The Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65, California Health and Safety Code 25249.5 *et seq*.) requires that the Governor cause to be published a list of those chemicals "known to the state" to cause cancer or reproductive toxicity. The Act specifies that "a chemical is known to the state to cause cancer or reproductive toxicity . . . if in the opinion of the state's qualified experts the chemical has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer or reproductive toxicity." The lead agency for implementing Proposition 65 is the Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency. The "state's qualified experts" regarding findings of carcinogenicity are identified as the members of the Carcinogen Identification Committee of the OEHHA Science Advisory Board (22 CCR 12301).

MX (3-chloro-4-(dichloromethyl)-5-hydroxy-2(*5H*)-furanone) was assigned a final priority of 'high' carcinogenicity concern and placed on the Final Candidate list of chemicals for Committee review on August 6, 1999. A public request for information relevant to the assessment on the evidence on the carcinogenicity of this chemical was announced in the *California Regulatory Notice Register* on August 6, 1999. No information was received as a result of this request.

This draft document *Evidence on the Carcinogenicity of MX* was developed to provide the Committee with relevant information for use in its deliberations. It reviews the available scientific evidence on the carcinogenic potential of MX. A public meeting of the Committee to discuss this evidence is scheduled for November 16, 2000. At this meeting it is expected that the Committee will render an opinion on whether MX has been clearly shown to cause cancer. Written public comment on the document should be submitted to OEHHA by October 24, 2000, in order to be considered by the Committee in advance of the meeting. During the November 2000 meeting, the public will have an opportunity to present verbal comments to the Committee.

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1 EXECUTIVE SUMMARY

MX (3-chloro-4-dichloromethyl-5-hydroxy-2(*5H*)-furanone) is a chlorination disinfection byproduct, which forms from the reaction of chlorine with humic acids in raw water. MX has been measured in drinking water samples in the United States (U.S.) and several other countries. Levels detected in drinking water were low and ranged from 2 to 67 ng/L.

MX induced cancer at multiple sites in male and female rats (Komulainen *et al.*, 1997; 2000). MX has not been tested for carcinogenic activity in mice. In male and female rats, MX induced thyroid gland follicular cell adenoma and carcinoma at all doses tested. Combined incidence of thyroid gland follicular cell adenoma and carcinoma reached 90 percent in the treated animals. Also in both sexes, statistically significant increased incidences relative to controls were observed for liver adenoma and carcinoma (combined) as well as adrenal gland cortical adenoma in the high-dose groups only. In females, MX significantly increased liver cholangioma and cholangiocarcinoma (combined) in multiple dose groups; in males, a dose-related trend for cholangioma was observed. Significantly increased incidences were also observed for benign and malignant mammary gland tumors in females. Marginally significant findings were observed for lymphoma and leukemia (combined) in females and pancreas Langerhans' cell adenoma and carcinoma (combined) in males.

MX is a direct acting mutagen and clastogen. The genotoxicity of MX has been investigated in approximately 100 publications. MX caused mutations in numerous strains of bacteria and was very potent in several test systems. MX induced mutations, chromosomal aberrations, sister chromatid exchanges (SCEs), strand breaks, or unscheduled DNA synthesis in human and other mammalian cells *in vitro*. MX exhibited mixed results in *in vivo* genotoxicity studies, following oral or intraperitoneal (i.p.) exposure of rodents to MX. Significant increases in strand breaks or alkali-labile sites, micronuclei or SCEs were observed in blood lymphocytes, kidney, stomach, jejunum, ileum, colon, duodenum, liver, lung, brain, spleen and bladder following oral or i.p. administration of MX to rodents. Several MX-derived DNA adducts have been characterized. The available data suggest that MX causes cancer primarily through a genotoxic mode of action, although the precise mechanism of carcinogenesis is not known. Available evidence suggests that MX may cause mutations through DNA adduction and misrepair and through an unusual, thermodynamic mechanism in which MX ionizes DNA bases.

MX was reported to induce cellular proliferation in the stomach of Wistar rats; a site at which tumors were not observed in carcinogenicity studies in the same rat strain. MX was also reported to act as a tumor promoter in an initiation/promotion study of the glandular stomach in Wistar rats. Studies of action as an initiator in classic initiation/promotion assays were not identified in the literature. Chlorinated and

brominated furanones, chemicals structurally similar to MX, also induced mutations in bacterial test systems.

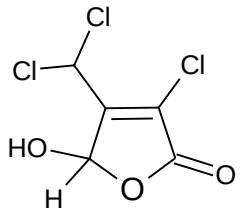
Thus, MX appears to be a multiple site carcinogen in male and female rats. MX has not been tested in mice. These carcinogenic findings are supported by extensive observations of mutagenicity and clastogenicity in test systems *in vitro* and in rats and mice *in vivo,* as well as suggestive evidence that MX may induce cellular proliferation or promote tumors in some tissues.

2 INTRODUCTION

2.1 Identity of MX (3-chloro-4-dichloromethyl-5-hydroxy-2(*5H*)-furanone)

A project to isolate and identify the mutagenic components formed in pulp chlorination was initiated in 1979 at the Pulp and Paper Research Institute of Canada (Holmbom *et al.*, 1981; Holmbom, 1990). Mutagenicity of the concentrates and fractions from the pulp chlorination process were tested for reverse mutations in *Salmonella typhimurium* strain TA100. Most of the mutagenicity was consistently found in one fraction, suggesting one primary mutagen. Because analytical techniques were not sufficiently advanced to easily identify the compound, the compound was called "Mutagen X" or "MX." After about a year of additional work, the chemical identity of MX was determined to be 3-chloro-4-(dichloromethyl)-5-hydroxy-2(*5H*)-furanone (Holmbom *et al.*, 1981).

Figure 1. Structure of MX



**Corrected August 31, 2000*

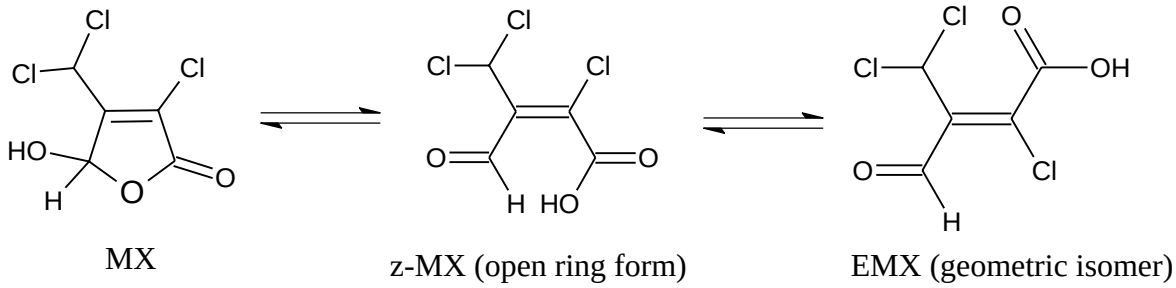
Molecular Formula: $C_5H_3Cl_3O_3$*
Molecular Weight: 217.4*
CAS Registry No.: 77439-76-0*
Chemical Class: chlorinated furanones, chlorinated water disinfection byproducts

Synonyms: mutagen X; chloro(dichloromethyl)-5-hydroxy-2(*5H*)furanone; 3-chloro-4-dichloromethyl-5-hydroxy-2-(*5H*)-furanone.

In acidic solutions MX is a furanone, but at physiological pH it exists primarily in a ring-open form, namely Z-2-chloro-3-(dichloromethyl)-4-oxo-butenoic acid (z-MX), a fraction of which undergoes isomerization to its geometric isomer (E)-2-chloro-3-

(dichloromethyl)-4-oxobutenoic acid (EMX) (Meier *et al.*, 1987a; Vartiainen *et al.*, 1991) (Figure 2). The solubility in water is high and is not appreciably affected by pH. The log n-octanol/water partition coefficient was estimated to be about 1.1 at pH 2 and changed with increasing pH to about -1.0 at pH 8 (Vartiainen *et al.*, 1991). At low pH, MX is stable while at higher pH it is less stable. For example, Vartiainen *et al.* (1991) observed that a 7.5 µg/mL solution of MX had a half life in water of 38 years at pH 2.8 and 7.4 days at pH 7.8.

Figure 2. Tautomeric forms of MX



2.2 Occurrence

The formation and occurrence of MX in drinking water and in effluents from chlorine bleaching processes have been reviewed elsewhere (Backlund *et al.*, 1988; Franzen, 1995; Langvik and Hormi, 1994). MX forms as a by-product from reactions of chlorine and humic acid material present in raw waters. MX has been detected in over 100 drinking water samples from municipal sources (Kronberg and Christman, 1989; Kronberg and Vartiainen, 1988; Smeds *et al.*, 1997; Suzuki and Nakanishi, 1990), at a prevalence of 83 to 100 percent in the samples taken. Thus, it is expected that millions of Californians are exposed daily to low levels of MX via chlorinated drinking water.

Concentrations of MX in drinking water samples taken from three U.S. cities were 2, 18 and 33 ng/L (Meier *et al.*, 1987b). Concentrations of MX ranged from not detectable to 67 ng/L among tap water samples taken from 26 Finnish localities (Kronberg and Vartiainen, 1988), from not detectable to 46 ng/L among samples taken from 35 Finnish localities and St. Petersburg, Russia (Smeds *et al.*, 1997), from three to nine ng/L among nine samples taken from five Japanese cities (Suzuki and Nakanishi, 1990), and from not detectable to 41 ng/L among drinking water samples taken from nine sites in the United Kingdom (Fawell and Horth, 1990). MX comprises a significant portion (7 to 67 percent) of the overall mutagenicity of chlorinated drinking water as measured in *Salmonella typhimurium* tester strain TA100 (see Section 3.3.1 Genetic Toxicology). Among approximately 70 Finnish drinking water samples, a linear correlation (r^2=0.73) between MX concentrations and the water's mutagenic potential in *Salmonella typhimurium* TA100 was observed (Vartiainen *et al.*, 1990).

Several researchers have studied the formation of MX from disinfection of raw water under different chlorination conditions. Formation of MX was favored during chlorination disinfection conditions of acidic reactions and high chlorine doses (Backland *et al.*, 1989a). However, in waters treated with excess chlorine at pH 9, no MX was detected. Ozone treatment prior to chlorination resulted in a slight decrease (~30 percent) in the concentration of MX formed (Backlund *et al.*, 1988). Backlund (1994) reported that the precursors of MX were destroyed in a dose-dependent manner by ozone or ozone in combination with ultraviolet (UV) irradiation, while UV irradiation alone resulted in a slight increase in the formation of MX and other mutagenic precursors. DeMarini *et al.* (1995) observed that ozonation alone did not increase the mutagenic potency of raw water. Ozonation prior to chlorination or chloramination reduced the mutagenic potency compared with chlorination or chloramination alone; and either chlorination or chloramination greatly increased the mutagenic potency of water extracts compared to raw or ozonated water. In test chlorination systems, addition of bromide to the water prior to chlorination resulted in formation of brominated analogues of MX and mixed chlorinated and brominated analogues of MX, which were also highly mutagenic (Fawell and Horth, 1990).

3 DATA ON MX CARCINOGENICITY

There is only one published report of carcinogenicity studies of MX. Komulainen *et al.* (1997) administered MX in the drinking water to male or female rats for two years, which resulted in the induction of tumors at multiple sites in both sexes of rats. There have been roughly 100 studies examining the genotoxicity of MX. These studies indicate that MX induces a wide variety of genetic damage including mutational and/or clastogenic effects in numerous strains of bacteria, in mammalian cells in culture, and in rodents *in vivo*.

3.1 Epidemiological Studies of Carcinogenicity

Although no data on long-term effects of human exposure to MX were found in the literature, there have been many reports of associations of chlorinated water consumption and increased risk of various cancers (reviewed in Cantor *et al.*, 1998; Craun, 1988; Koivusalo *et al.*, 1994; 1995; 1998; Morris *et al.*, 1992; Morris, 1995; U.S. EPA, 1998a). A meta-analysis of the available epidemiological studies suggested an association between chlorinated disinfection byproducts and increased risk of bladder and rectal cancer (Morris *et al.*, 1992). Other studies have observed associations of drinking water mutagenicity and lymphomas, and cancers of the pancreas, kidney, stomach, and bladder (Koivusalo *et al.*, 1994; 1995; 1998). The U.S. Environmental Protection Agency (U.S. EPA, 1998a) has also extensively reviewed the available epidemiological studies and concluded that the strongest association between consumption of chlorinated drinking water and human cancers is with bladder cancer. U.S. EPA (1998a) suggests that further studies are warranted.

3.2 Carcinogenicity Studies in Animals.

The carcinogenicity of MX has been investigated in male and female rats given the compound in drinking water (Komulainen *et al.*, 1997). Tumor incidences for specific benign or malignant tumors were reported in the original study publication (Komulainen *et al.*, 1997). Combined frequencies for benign and malignant tumors of similar tumor types were kindly provided to OEHHA by the study authors (Komulainen *et al.*, 2000). Statistically significant increases in the incidences of liver adenoma and carcinoma (combined), adrenal gland cortical adenoma, thyroid follicular cell adenoma, and thyroid follicular cell carcinoma were observed for both male and female MX-treated rats relative to controls. Increased incidences of mammary gland adenocarcinoma, mammary gland fibroadenoma, and liver cholangioma also were observed among MX-treated female rats relative to controls. The thyroid gland was the most sensitive tissue for both sexes. In addition, dose-related increases, significant only by trend test, were reported for incidences of various tumors of the skin, pancreas and liver in male rats, and the hematopoietic system of female rats (see below). MX has not been tested for carcinogenic activity in mice.

Rat Drinking Water Studies: Komulainen et al., 1997

Male and female Wistar rats (50 animals per dose group, five weeks of age) were administered MX in their drinking water at mean concentrations of 0, 5.9, 18.7 or 70.0 µg/mL for 104 weeks. The authors reported that the water concentrations and consumption rates resulted in average daily doses of 0, 0.4, 1.3 or 5.0 mg/kg body weight for male rats and 0, 0.6, 1.9 or 6.6 mg/kg body weight for female rats. The highest doses tested (5.0 and 6.6 mg/kg-day for males and females, respectively) were originally chosen because they represented the approximate lowest-observed-effects level from an earlier range-finding subchronic study (Vaittinen *et al*., 1995).

The doses of MX administered in these carcinogenicity studies did not cause overt toxicity in the rats. No MX-related clinical signs were observed in treated animals, and mortality did not differ between the groups. Food consumption and body-weight gain were similar across dose groups, although a slight but statistically significant reduction in body weight was observed among high-dose males and female rats. Water consumption was decreased in both sexes in a dose-dependent manner, which the authors attributed to the lack of palatability of the MX dose formulations. However, the authors commented that the reduced water consumption among treated animals did not notably affect liquid balance since urine volumes did not differ among controls and treated groups at the end of the studies. Rats surviving to 104 weeks were sacrificed. All animals in the studies were autopsied.

MX induced cancer at multiple sites in male and female rats (Komulainen *et al.*, 1997; 2000). Summaries of the tumor incidences are presented in Table 1 for male rats and Table 2 for female rats. In male rats, MX induced statistically significant increases relative to controls (i.e., pairwise comparisons) in the incidences of thyroid gland

follicular cell adenoma and carcinoma in all doses tested. Combined incidence of thyroid gland follicular cell adenoma and carcinoma reached 90 percent in the treated animals. In addition, statistically significant increased incidences relative to controls were observed for liver adenoma and carcinoma (combined) as well as adrenal gland cortical adenoma in the high dose group only. Statistically significant dose-related increases, as determined by one-sided trend tests, were observed among the male rats for basal cell skin tumors (malignancy status not determined), lung adenoma, liver adenoma, liver adenoma and carcinoma (combined), liver cholangioma, pancreas Langerhans' cell adenoma, pancreas Langerhans' cell adenoma and carcinoma (combined), adrenal gland cortical adenoma, thyroid follicular cell adenoma, thyroid follicular cell carcinoma, and thyroid follicular cell adenoma and carcinoma (combined). Additionally, there appeared to be slight increases in the incidences of lymphoma and leukemia (combined) among all groups of MX-treated male rats compared to the controls; however, these findings failed to reach statistical significance either by pairwise or trend tests.

In female rats, MX induced statistically significant increases relative to controls (pairwise comparisons) in the incidences of thyroid gland follicular cell adenoma, adenoma and carcinoma (combined), and carcinoma (high dose only) as well as liver cholangioma, liver cholangioma and cholangiocarcinoma (combined) in multiple dose groups. Combined incidence of thyroid gland follicular cell adenoma and carcinoma reached 94 percent in the treated animals. Statistically significant increased incidences relative to controls were also observed for mammary gland adenocarcinoma, mammary gland fibroadenoma, liver adenoma, and adrenal gland cortical adenoma in the high-dose groups only. Statistically significant dose-related increases, as determined by one-sided trend tests, were observed among the female rats for mammary gland adenocarcinoma, mammary gland fibroadenoma, mammary gland adenoma and adenocarcinoma (combined), lymphoma and leukemia (combined), liver adenoma, liver adenoma and carcinoma (combined), liver cholangioma, adrenal gland cortical adenoma, thyroid follicular cell adenoma, thyroid follicular cell carcinoma, and thyroid follicular cell adenoma and carcinoma (combined).

Thus, MX appears to be a multisite carcinogen, eliciting increases in the frequency of tumors of the thyroid gland, liver, and adrenal gland in both sexes of rats, and mammary gland in females (Komulainen *et al.*, 1997; 2000). MX treatment induced the strongest tumorigenic response in the thyroid glands in both male and female rats, with increases in all dose groups compared to controls. In both sexes, the incidences of thyroid follicular cell adenoma/carcinoma (combined) approached 100 percent in the high dose groups. Other associations, which were significant only by trend test, suggested possible tumorigenic activity in the skin, lung, and pancreas of male rats as well as the hematopoietic system of female rats.

Table 1. Tumors in male Wistar rats receiving MX in drinking water for 104 weeks (Komulainen *et al.*, 1997; 2000)

Tissue	MX, mg/kg-d				p-value
	Control	0.4	1.3	5.0	(trend)[d]
Skin, subcutaneous tissue					
basal cell tumor	1/50	0/50	1/50	3/50	0.0314
Lung, alveolar and					
bronchiolar adenoma	2/50	1/50	1/50	7/50	0.0015
Lymphoma and leukemia	0/50	3/50	4/50	3/50	0.1527
Liver					
carcinoma	0/50	0/50	2/50	1/50	0.1605
adenoma	0/50	1/50	2/50	4/50	0.0142
adenoma or carcinoma	0/50	1/50	3/50	5/50[a]	0.0066
cholangioma	0/50	0/50	1/50	4/50	0.0009
Pancreas, Langerhans' cell					
carcinoma	4/50	3/50	5/50	4/50	0.3769
adenoma	5/50	8/50	8/50	12/50	0.0116
adenoma or carcinoma	9/50	11/50	12/50	15/50	0.0312[d]
acinar cell adenoma	2/50	3/50	2/50	4/50	0.1243
Adrenal gland					
cortical adenoma	5/50	2/50	7/50	14/50[a]	0.0001
Thyroid gland					
follicular carcinoma	0/49	1/50	9/50[b]	27/49[c]	<0.0001
follicular adenoma	2/49	20/50[c]	34/50[c]	21/49[c]	0.0045
follicular adenoma or carcinoma	2/49	20/50[c]	38/50[c]	44/49[c]	<0.0001

[a] Significantly different from control animals by pairwise Fisher Exact Test, $p \leq 0.05$

[b] Significantly different from control animals by pairwise Fisher Exact Test, $p \leq 0.01$

[c] Significantly different from control animals by pairwise Fisher Exact Test, $p \leq 0.001$

[d] Results of one-sided trend test reported by Komulainen *et al.* (1997; 2000) using a statistical program TRIAL2 based on methods described by Peto *et al.* (1980). OEHHA obtained similar p-values using a Mantel-Haenszel trend test. One notable difference was that the trend in the combined incidence of pancreatic tumors among male rats was not statistically significant using the Mantel-Haenszel trend test (p=0.09).

Table 2. Tumors in female Wistar rats receiving MX in drinking water for 104 weeks (Komulainen *et al.*, 1997; 2000)

Tissue	MX, mg/kg-d				p-value
	Control	0.6	1.9	6.6	(trend)[d]
Mammary gland					
adenocarcinoma	3/50	2/50	5/50	11/50[a]	0.0012
fibroadenoma	23/50	25/50	32/50	34/50[a]	0.0090
adenoma	0/50	0/50	3/50	1/50	0.1641
adenoma or adenocarcinoma	3/50	2/50	7/50	12/50[b]	0.0008
atypic hyperplasia	0/50	0/50	3/50	2/50	0.1032
atypic hyperplasia, adenoma, or adenocarcinoma	3/50	2/50	9/50	13/50[b]	0.0004[e]
Lymphoma and leukemia	1/50	1/50	2/50	4/50	0.0474
Liver					
carcinoma	1/50	1/50	3/50	0/50	0.7011
adenoma	1/50	1/50	1/50	10/50[b]	<0.0001
adenoma or carcinoma	2/50	2/50	4/50	10/50[b]	0.0001
cholangiocarcinoma	1/50	0/50	0/50	2/50	0.0828
cholangioma	0/50	4/50	10/50[c]	33/50[c]	<0.0001
cholangioma or cholangiocarcinoma	1/50	4/50	10/50[c]	34/50[c]	<0.0001
Adrenal glands					
cortical adenoma	5/50	10/50	12/50	16/50[b]	0.0098
Thyroid gland					
follicular carcinoma	1/50	3/49	6/50	22/50[c]	<0.0001
follicular adenoma	4/50	16/49[b]	36/50[c]	36/50[c]	<0.0001
follicular adenoma or carcinoma	5/50	18/49[b]	38/50[c]	47/50[c]	<0.0001
C-cell carcinoma	0/50	0/49	0/50	1/50	0.0926
C-cell adenoma	11/50	11/49	10/50	16/50	0.0631

[a] Significantly different from control animals by pairwise Fisher Exact Test, $p \leq 0.05$

[b] Significantly different from control animals by pairwise Fisher Exact Test, $p \leq 0.01$

[c] Significantly different from control animals by pairwise Fisher Exact Test, $p \leq 0.001$

[d] Results of one-sided trend test reported by Komulainen *et al.* (1997; 2000) using a statistical program TRIAL2 based on methods described by Peto *et al.* (1980). OEHHA obtained similar p-values using a Mantel-Haenszel trend test.

[e] Mantel-Haenszel trend test. Trend test not reported by Komulainen *et al.* (2000).

3.3 Other Relevant Data

In addition to the reported animal bioassays, other data related to the possible carcinogenicity of MX are available. These include studies of genetic toxicity, pharmacokinetics and metabolism, structure-activity comparisons, cellular proliferation, tumor promotion, and mechanism of action.

3.3.1 Genetic Toxicology

The genotoxicity of MX has been extensively studied (Tables 3 and 4). MX caused DNA damage in bacteria, plants, fish, shellfish, mammalian cells *in vitro* and rodents *in vivo*.

In vitro genotoxicity studies

MX induced mutations in a variety of bacterial strains and other short-term assays, with high potency in some test systems (Table 3). MX was one of the most potent compounds ever tested in *Salmonella typhimurium* strain TA100. The reported number of revertants/nmol MX was high: 5,600 (Kronberg *et al.*, 1988), 6,300 (Ishiguro *et al.*, 1988), or 13,000 (Meier *et al.*, 1987b). MX appears to be a direct acting mutagen; addition of human placental S9 or rat liver S9 reduced the mutagenicity of MX (Cozzie *et al.*, 1993; Ishiguro *et al.*, 1987; 1988; Vartiainen *et al.*, 1989). In the forward-mutation assay for 8-azaguanine resistance in *Salmonella* TM677, MX was observed to be twice as potent as aflatoxin B1, making MX the most potent mutagen tested in this assay (DeMarini *et al.*, 1995). Similarly, MX induced prophage λ (plaque-forming units) with or without exogenous activation enzymes (S9). The potency of MX was in the same range as topoisomerase II poisons, oxolinic acid and nalidixic acid, making MX among the most potent inducers of prophage λ identified in the Microscreen assay (DeMarini *et al.*, 1995).

As described earlier (Section 2.2), MX is present in chlorinated drinking water. Among approximately 70 Finnish drinking water samples, a linear correlation (r^2=0.73) between MX concentrations and the water's mutagenic potential in *Salmonella typhimurium* tester strain TA100 was observed (Vartiainen *et al.*, 1990). Numerous investigators have reported positive mutagenicity of drinking water samples in *Salmonella typhimurium* strains TA97, TA98 or TA100 and have attributed a portion of the mutagenicity to MX (Backlund, 1994; Backlund *et al.*, 1988; 1989a; 1989b; Fawell and Horth, 1990; Hemming *et al.*, 1986; Kronberg *et al.*, 1988; Kronberg and Christman, 1989; Kronberg and Franzen, 1993; Kronberg and Vartiainen; 1988; Langvik *et al.*, 1991b; 1994; Meier *et al.*, 1987b; Smeds *et al.*, 1997; Suzuki and Nakanishi, 1990; Vartiainen, 1989; Vartiainen *et al.*, 1990).

In fact, MX frequently comprises a significant portion of the overall mutagenicity of chlorinated drinking water as measured in *Salmonella typhimurium* TA100. Smeds *et al.* (1997) analyzed drinking water samples from 35 locations in Finland and St. Petersburg, Russia. They reported that MX accounted for up to 67 percent of the overall

mutagenicity (*Salmonella typhimurium* TA100) of the water samples. These findings are consistent with previous reports of the percentage of total drinking water mutagenicity (*Salmonella typhimurium* TA100) attributable to MX: 15 to 30 percent among three samples taken from three U.S. cities (Meier *et al.*, 1987b), 7 to 23 percent among nine samples taken from five Japanese cities (Suzuki and Nakanishi, 1990), and 15 to 57 percent among samples taken from 26 Finnish localities (Kronberg and Vartiainen, 1988).

DeMarini *et al.* (1995) compared the mutational spectra induced by MX to spectra induced by different water samples (raw, chlorinated, ozonated, or chloraminated) and by other aromatic mutagens. In *Salmonella typhimurium* TA100, MX and the chlorinated water extracts exhibited similar mutational spectra, which were predominated by GC $\rightarrow$ TA transversions. DeMarini *et al.* (1995) estimated that approximately 20 percent of the TA100 mutagenicity was due to MX and suggested that other chlorinated organic byproducts, such as halogenated polyaromatic hydrocarbons, produced lesions similar to MX.

MX's high affinity for protein and other cellular nucleophiles is likely to reduce the mutagenic potency in mammalian cells or in whole animal test systems. When other nucleophiles such as SO_2 (HSO_3^-, SO_3^{2-}), $S_2O_3^{2-}$, pyrrolidine, L-cysteine, glutathione or bovine serum albumin were added to the reaction mixture, bacteria mutagenicity was reduced (Ishiguro *et al.*, 1987; Cozzie *et al.*, 1993; Watanabe *et al.*, 1994). MX was observed to bind strongly to albumin (Haataja *et al.*, 1991). Kinae *et al.* (1992) reported that addition of human serum to the reaction media eliminated MX's mutagenicity in bacteria (*Salmonella typhimurium* TA100); however, addition of human saliva did not diminish MX's mutagenicity.

Although glutathione reduced MX's mutagenic potential in *Salmonella typhimurium* TA100, addition of glutathione increased the ability of MX to induce strand breaks in the ϕX174 plasmid assay (LaLonde and Ramdayal, 1997), suggesting a separate, glutathione-mediated mutagenic pathway.

MX induces several types of mutations, including base-pair substitutions, as evident by positive mutagenicity in *Salmonella typhimurium* strains TA100 and TA1535, as well as frameshift mutations, as evident by mutagenicity in *Salmonella typhimurium* strains TA97 and TA98 (Niittykoski *et al.*, 1995). Several researchers have investigated the mutational spectra induced by MX in bacterial and mammalian cells (DeMarini *et al.*, 1992; 1995; Hyttinen *et al.*, 1995; 1996; Knasmüller *et al.*, 1996; Niittykoski *et al.*, 1995). In *Salmonella typhimurium* strain TA100, MX induced a base pair substitution, namely a GC $\rightarrow$ TA transversion, in 60 to 87 percent of the colonies examined (DeMarini *et al.*, 1992; Hyttinen *et al.*, 1995). A hotspot (a two- to three-fold preference) was observed in the second position of the *hisG46* target CCC codon (DeMarini *et al.*, 1992; Hyttinen *et al.*, 1995). A predominance of GC $\rightarrow$ TA transversions were also observed in *Salmonella typhimurium* strains TA100, TA1535, TA1950 and TP2428 treated with MX

Table 3. Genotoxicity of MX in *in vitro* test systems

Test System	Result	References
Purified DNA		
Abasic sites and strand breaks following exonuclease post-treatment (supercoiled PM2 DNA)	+	Hyttinen and Jansson, 1995
Strand breaks (plasmid ϕX174 assay)	+	LaLonde and Ramdayal, 1997
Bacteria		
Reverse mutation *Salmonella typhimurium* strains TA97, TA98, TA100, TA102, TA104[a], TA1535, TA1538, TA1950, TP2428, UTH8413, UTH8414,YG7119[b], YG7113[b], YG112[b]	+	Alhonen-Raatesalmi and Hemminki, 1991; Backlund *et al.*, 1990; Clark and Chipman, 1995; Cozzie *et al.*, 1993; DeMarini *et al.*, 1992; 1995; Fawell and Horth, 1990; Franzen, 1995; Franzen *et al.*, 1998a[a]; Furihata *et al.* (1992); Haatja *et al.*, 1991; Hemming *et al.*, 1986; Hyttinen *et al.*, 1995; Ishiguro *et al.*, 1987; 1988; Jansson *et al.*, 1995; Kinae *et al.*, 1992; Knasmüller *et al.*, 1996; Kronberg *et al.*, 1988; LaLonde *et al.*, 1991a; 1991b; 1991c; 1997; Lampelo *et al.*, 1989; Langvik *et al.*, 1991a; Meier *et al.*, 1987b; 1989; Schenck *et al.*, 1989; 1990; Taylor *et al.*, 1995; Tikkanen and Kronberg, 1990; Suzuki and Nakanishi, 1995; Vartiainen *et al.*, 1989; 1991; Yamada *et al.*, 1997[b]
Forward Mutation *Salmonella typhimurium* strain TM677	+	DeMarini *et al.*, 1992; 1995

Table 3. Genotoxicity of MX in *in vitro* test systems (continued)

Test System	Result	References
Reversion Mutation *Escherichia coli* strains WP2, CC101, CC102, CC103, CC104, CC105, CC106, CC107, CC108, CC109, CL101P, CL102P, CL103P, CL104P, CL105P, ZA500, ZA4107, ZA4108, ZA4109, *ZA2102, ZA2104, ZA2107, ZA2108, ZA2109, ZA21110, ZA2111, ZA5102, ZA5104, ZA5108, ZA5109*	+	Lu and Chen, 1992; Lu *et al.*, 1995; Watanabe-Akanuma and Ohta, 1994; Watanabe-Akanuma *et al.*, 1997; Watanabe *et al.*, 1994.
DNA damage (chromotest) *Escherichia coli* strain PQ 37	+	Tikkanen and Kronberg, 1990
Prophage λ induction *Escherichia coli* system	+	DeMarini *et al.*, 1995
DNA damage (differential DNA repair) *Escherichia coli* strain K-12	+	Fekadu *et al.*, 1994
Mammalian cells (in vitro) (human-derived cells are shown in bold)		
Mutation, mouse L5178Y lymphoma cell forward mutation assay (thymidine kinase locus)	+	Harrington-Brock *et al.*, 1995
Mutation, Chinese hamster ovary cell forward mutation assay (hypoxanthine phosphoribosyl transferase (HPRT) locus or the Na/K ATPase locus)	+	Hyttinen *et al.*, 1996; Jansson and Hyttinen, 1994; Jansson *et al.*, 1995; Mäki-Paakkanen *et al.*, 1994
Mutation, Chinese hamster V79 cell forward mutation assay (HPRT locus)	+[c]	Matsumura *et al.*, 1994
DNA strand breaks (DNA alkaline unwinding assay) **human lymphoblastoid cell line CCRF-CEM.**	+	Chang *et al.*, 1991

Table 3. Genotoxicity of MX in *in vitro* test systems (continued)

Test System	Result	References
DNA damage (strand breaks and alkali-labile sites, alkaline elution or comet assay) **human lymphocytes** **human peripheral blood mononuclear cells** (resting or growth stimulated) **human LLC-PK1 renal proximal tubular epithelial cells** **HL-60 cells** pig kidney cells rat hepatocytes rat testicular cells Chinese hamster V79 cells	+	Brunborg *et al.*, 1991; 1997; Hodges *et al.*, 1997; Holme *et al.*, 1999; Marsteinstredet *et al.*, 1997
DNA strand breakage (fluorometric analysis of DNA unwinding) **human lymphocytes**	+	Nunn and Chipman, 1994
Chromosome aberrations (chromatid and chromosome) Chinese hamster ovary cells mouse L5178Y lymphoma cells rat peripheral lymphocytes	+	Harrington-Brock *et al.*, 1995; Jansson *et al.*, 1993; Mäki-Paakkanen *et al.*, 1994; Meier *et al.*, 1987b
Micronuclei Chinese hamster ovary cells mouse L5178Y lymphoma cells	+	Le Curieux *et al.*, 1999; Mäki-Paakkanen *et al.*, 1994
Sister chromatid exchanges (SCEs) Chinese hamster ovary cells rat peripheral lymphocytes;	+	Jansson *et al.*, 1993; Mäki-Paakkanen *et al.*, 1994

Table 3. Genotoxicity of MX in *in vitro* test systems (continued)

Test System	Result	References
Unscheduled DNA synthesis mouse hepatocytes rat hepatocytes	+	Nunn *et al.*, 1997; Le Curieux *et al.*, 1999

[a] Assay conducted on the open form of MX, i.e., (Z)-2-chloro-3(dichloromethyl)-4-oxobutenoic acid

[b] YG7119, YG112 and YG7113 are derivatives of *Salmonella typhimurium* tester strain TA100 which contain different inactivating deletions in the genes encoding the DNA repair enzyme, O^6-methylguanine DNA methyltransferase.

[c] MX induced mutations in the V79 cells when diluted in a buffered salt solution, but did not induce mutations when diluted in tissue culture medium.

and several related chlorohydroxyfuranones (Knasmüller *et al.*, 1996) and were observed in *lacZ*$^-$ mutants in *Escherichia coli* strains CL101P, CL102P, CL103P, CL104P and CL105P (Lu *et al.*, 1995). Lu *et al.* (1995) observed that in addition to the predominant GC → TA transversions a significant proportion (38 percent) of the total DNA modifications in the *lacZ*$^-$ mutants were AT → CG transversions.

In *Salmonella typhimurium* TA98, a bacterial strain sensitive to frameshift mutations, MX produced two-base deletions in 40 to 70 percent of mutants and complex frameshifts (frameshifts with an adjacent base substitution, which were mostly GC → TA transversions) in about 30 to 50 percent of mutants (DeMarini *et al.*, 1992; 1995). According to DeMarini *et al.* (1995) no other compound or mixture is known to induce such high frequencies of complex frameshifts. MX-induced revertants had a two-base deletion in either GC or CG in the CGCCGCG hotspot of the *hisD3052* allele (DeMarini *et al.*, 1992; 1995).

In mammalian cells, MX induced mutations that were primarily GC → TA transversions in the HPRT locus of Chinese hamster ovary cells. However, some mutations contained AT → TA transversions, deletions of single GC base pairs (i.e., frameshifts off by one base), or had large deletions or insertions in the cDNA (which the authors suggested could be explained by splicing errors) (Hyttinen *et al.*, 1996).

The mutations in mammalian cells and bacterial involving GC → TA transversions are consistent with the 'A rule' that DNA polymerases preferentially insert adenine nucleotides in positions opposite non-instructional lesions, such as abasic sites (Strauss, 1991). Additionally, the other predominant mutation in mammalian cells (i.e., single base deletions within runs of GC base pairs) may be explained by failure of the translesional DNA synthesis followed by dislocation of the DNA template structure (Hyttinen *et al.*, 1996; Strauss, 1991).

Table 4. Genotoxicity of MX *in vivo*

Endpoint	Species	Tissue	Dose Regimen (hours between dosing and sacrifice)	Result	References
Micronuclei	mice	Polychromatic erythrocytes	oral gavage for 14 days at 64 mg/kg body weight	-	Meier *et al.*, 1996
Micronuclei	mice	Polychromatic erythrocytes	i.p., single dose at 0, 4.4 or 8.8 mg/kg (24 h)	-	Tikkanen and Kronberg, 1990
Micronuclei	mice	Duodenum forestomach Duodenum forestomach	oral gavage, single dose at 0.28, 0.37 or 0.46 mmol/kg (24 h) oral gavage, single dose at 0.28, 0.37 or 0.46 mmol/kg (study repeated)	+ + + +	Daniel *et al.*, 1991
Micronuclei (or aberrations, type not specified)	mice	bone marrow liver gastrointestinal tract stomach	oral gavage, single dose at 144 mg/kg (time to sacrifice not reported)	- - - -	Fawell and Horth, 1990
Micronuclei	mice	Polychromatic erythrocytes	oral gavage for two days at 22, 45 or 90 mg/kg	-	Meier *et al.*, 1987b
Micronuclei	rats	Polychromatic erythrocytes	drinking water for 104 weeks at 0, 5.9, 18.7 or 70.0 mg/L	-	Jansson, 1998
Micronuclei	rats	Peripheral blood lymphocytes	oral gavage, three days at doses of 25 to 150 mg/kg	+	Mäki-Paakkanen and Jansson, 1995

Table 4. Genotoxicity of MX *in vivo* (continued)

Endpoint	Species	Tissue	Dose Regimen (hours between dosing and sacrifice)	Result	References
SCEs	rats	Peripheral blood lymphocytes	oral gavage, five days per week for 14 to 18 weeks, doses 30 and 45-75 mg/kg	+	Jansson *et al.*, 1993
SCEs	rats	Peripheral blood lymphocytes kidney	oral gavage, three days at doses of 25 to 150 mg/kg	+ +	Mäki-Paakkanen and Jansson, 1995
DNA damage (alkaline elution)	mice	Hematopoietic system liver lung kidney brain spleen stomach jejunum ileum colon bladder	oral gavage, single dose at 100 mg/kg (0, 1, 3, 6 or 24 h)	- + + + + + + + + + +	Sasaki *et al.*, 1997a
DNA damage (alkaline elution)	mice	Liver kidney spleen colon	i.p., single dose at 40 or 80 mg/kg (1 h), or intra-rectal intubation, single dose at 40 or 80 mg/kg (1 h)	-[a] - - -	Holme *et al.*, 1999

Table 4. Genotoxicity of MX *in vivo* (continued)

Endpoint	Species	Tissue	Dose Regimen (hours between dosing and sacrifice)	Result	References
DNA damage (alkaline elution)	rats	pyloric mucosa of the stomach	oral gavage, single dose at 0, 10, 20, or 48 mg/kg (2 h)	+	Furihata *et al.*, 1992
DNA damage (alkaline elution)	rats	small intestine large intestine stomach liver kidney lung bone marrow urinary bladder testis	oral gavage, single dose at 18, 63 or 125 mg/kg (1 h), or i.p., single dose at 18 mg/kg (1 h)	+ - - - - - - - -	Brunborg *et al.*, 1990; 1991
DNA damage (differential DNA repair, as measured in two strains of *Escherichia coli* K-12 isolated from various organs)	mice	Stomach lung intestine liver kidney spleen	single oral dose at 0, 4.3, 13, 40 or 200 mg/kg (2 h)	+ + + + + +	Fekadu *et al.*, 1994

Table 4. Genotoxicity of MX *in vivo* (continued)

Endpoint	Species	Tissue	Dose Regimen (hours between dosing and sacrifice)	Result	References
Replicative DNA synthesis	rats	pyloric mucosa of the stomach	oral gavage, single dose at 0, 10, 30 or 60 mg/kg (16 h)	+	Furihata *et al.*, 1992
Unscheduled DNA synthesis	mice	Hepatocytes	oral gavage, single dose at 100 mg/kg (3 or 16 h)	+[b]	Nunn *et al.*, 1997

[a] Holme *et al.* (1999) observed dose-related DNA damage (as measured by alkaline elution method) in the liver and kidney when mice were pretreated with DNA repair enzyme inhibitors, but not without pretreatment.

[b] An increased net nuclear grain count was observed three hours after administration of MX, which is usually considered a positive finding. However, the authors did not conclude that this increase represented a positive finding since the effect was not strong and the increase was not observed 16 hours after dosing (see text).

In vivo genotoxicity studies

Numerous investigators have studied the genotoxic potential of MX in whole animal systems (Table 4). Some studies observed clear increases in DNA damage (e.g., SCEs, micronuclei, differential DNA repair, single strand breaks, and alkali-labile sites) while other studies did not. Many of the negative genotoxicity findings can be explained by the apparent tissue selectivity of MX-induced DNA damage. MX caused genetic damage to a wide range of tissues but did not appear to affect the bone marrow (Sasaki *et al.*, 1997a). Thus, negative results reported by Meier *et al.* (1987a; 1996), Tikkanen and Kronberg (1990), and Jansson (1998) might be expected since these investigators examined alterations in bone marrow or polychromatic erythrocytes (an indicator of bone marrow damage).

A second reason for the differences observed among studies might be due to the time kinetics of DNA damage and repair and the time at which the DNA damage was examined. Sasaki *et al.* (1997a) observed significant tissue specificity and variability in the kinetics of DNA damage. For example, liver DNA damage was evident one hour after administration but had returned to background levels by three hours after administration. Several tissues of the urinary and gastrointestinal tract exhibited DNA damage that persisted for 24 hours following MX administration. As seen in Table 4, the *in vivo* studies that reported negative findings in tissues other than the bone marrow were single-dose studies in which the animals were sacrificed one hour after administration of MX (Brunborg *et al.*, 1990; 1991; Holme *et al.*, 1999), or in one case the duration was not reported (Fawell and Horth, 1990). Of course it is also possible that the differences in the studies findings could be due to chance. Some authors have suggested that the negative findings reflect a low genotoxic potential for MX *in vivo* (Holme *et al.*, 1999; Jansson, 1998). However, the evidence taken together suggests that MX likely exerts DNA damage to a wide range of tissues *in vivo* (Table 4). The *in vivo* studies of MX are briefly summarized below.

Micronuclei

Meier *et al.* (1987a) treated groups of male and female Swiss Webster mice with MX by oral gavage at doses of 22, 45 or 90 mg/kg-day body weight for two days. No increases were observed relative to vehicle controls in the frequency of micronuclei in polychromatic erythrocytes 48 or 72 hours following exposure. Similarly, Meier *et al.* (1996) treated $B6C3F_1$ mice with MX for 14 days by oral gavage and did not observe a significant increase in the frequency of micronuclei in the peripheral blood polychromatic erythrocytes.

Fawell and Horth (1990) treated five male and five female CD-1 mice with a single oral dose of MX at 144 mg/kg body weight. The time between exposure and sacrifice was not reported. No micronuclei or chromosomal aberrations (type not specified) were observed in the bone marrow, liver, bladder, stomach, duodenum, jejunum, ileum, colon or rectum.

Cyclophosphamide, which was used as a positive control, elicited clear effects in several tissues.

Tikkanen and Kronberg (1990) administered a single i.p. injection of MX to five male and five female NMRI mice at doses of 0 (vehicle), 4.4 or 8.8 mg/kg body weight. No increases in polychromatic erythrocytes were observed 24 hours after exposure.

Daniel *et al.* (1991) treated male $B6C3F_1$ mice (ten animals per group) with a single oral dose by gavage at doses of 0, 37.8, 50.0 or 62.2 mg MX/kg body weight. Animals were sacrificed 24 hours after treatment. Tissues from the forestomach, duodenum and proximal colon were assayed for micronuclei. Dose-related increases in micronuclei were observed in the forestomach and duodenum by trend test. By pairwise analysis (Fisher Exact Test), the increased incidences were statistically significant for the two highest doses only. Daniel *et al.* (1991) repeated the experiment using the same number of animals per group and the same dose levels. They obtained similar results as in the first experiment.

Jansson (1998) examined bone marrow smears taken from male and female rats treated with MX in the drinking water for two years (Komulainen *et al.*, 1997) (See Section 3.2 Carcinogenicity Studies in Animals). Samples from 15 control animals and from MX-treated rats (ten/sex/dose) were examined. A slight dose-related increase in micronuclei in polychromatic erythrocytes was observed among male rats, but this increase was not statistically significant. No increases in micronuclei were observed among female rats. It is unclear why the author selected the bone marrow for study, since the animal bioassay results and prior genotoxicity studies failed to suggest bone marrow as a target organ for MX.

SCEs

Jansson *et al.* (1993) administered MX to male and female Han:Wistar rats by oral gavage, five days per week for 14 to 18 weeks. Male and female rats were divided into three dose groups: a vehicle control group, a low dose group (30 mg/kg body weight) and a high dose group (45 mg/kg for seven weeks, followed by 60 mg/kg for two weeks and finally 75 mg/kg-body weight for five weeks). Statistically significant dose-related increases in the frequency of SCEs in the peripheral blood lymphocytes were observed in both male and female rats. In an extension of this study, Mäki-Paakkanen and Jansson (1995) observed increases in SCEs ($p<0.001$, trend) and micronuclei ($p<0.004$, trend) in the peripheral blood lymphocytes of male Han:Wistar rats administered gavage doses of 0, 25, 50, 100 or 150 mg/kg body weight for three consecutive days. Statistically significant increases in SCEs in kidney cells of the rats were also observed.

DNA damage as measured by alkaline elution, unscheduled DNA synthesis and other techniques

Brunborg *et al.* (1990; 1991) administered a single gavage dose of MX at 0, 18, 63 or 125 mg/kg body weight or a single i.p. injection of 18 mg/kg body weight to male Wistar rats, which had fasted for two days prior to treatment. The number of rats used in the study was not reported. Organs were removed and assayed one hour after exposure. DNA damage (presumably single strand breaks or alkali-labile sites) was measured by an automated alkaline elution assay test system in which the DNA was loaded onto a filter and the fraction of DNA that eluted from the filter was counted by a fluorochrom detector. An increase in DNA damage was reported in the small intestine of rats in the high-dose group treated orally. However, the authors concluded that there was no evidence of induced DNA damage. Ten other organs were assayed: large intestine, stomach, liver, kidney, lung, bone marrow, urinary bladder and testis. For these tissues, elution rates of the DNA from MX-treated rats did not differ significantly from controls.

Furihata *et al.* (1992) administered a single oral gavage dose to male F344/Du Crj rats at concentrations of 0, 10, 20 or 48 mg MX/kg body weight. Dose-related increases in DNA single strand breaks (as measured by alkaline elution) were observed over all doses in the pyloric stomach mucosa. In pairwise tests, only the highest dose reached statistical significance. Furihata *et al.* (1992) also administered a single oral dose to rats at concentrations of 0, 10, 30 or 60 mg MX/kg body weight and observed dose-related increases in replicative DNA synthesis in the pyloric stomach mucosa. In pairwise tests, the increases were statistically significant in all dose groups relative to controls ($p<0.01$). Furihata *et al.* (1992) also studied the time-dependent induction of ornithine decarboxylase (ODC) activity in the pyloric mucosa of the stomach of rats given an oral dose of 60 mg MX/kg. Tissue samples were taken 0, 4, 8, 16 and 24 hours following administration of MX. ODC activity was induced by approximately 400-fold and reached maximal levels at 16 hours after administration. Although ODC activity may not be related to MX genotoxicity, these findings do suggest that studies that sacrificed the animals soon after exposure (e.g., after one hour) may not have observed the full potential of MX's bioactivity.

Fekudu *et al.* (1994) used an animal-mediated assay to study the genotoxicity of MX in mice. As indicators of DNA damage, two strains of *E. coli* K-12 that differed in their DNA repair capability (uvrB/recA versus uvr+/rec+) were employed. Exposure to a genotoxin results in a preferential reduction of the viability of the repair-deficient strain relative to the repair-proficient strain. Mixtures of the two indicator strains were injected into the tail vein of Swiss albino mice. Two hours after injection of the indicator strains, the mice were given a single oral dose by gavage at concentrations of 0, 4.3, 13, 40 or 200 mg MX/kg body weight. The two strains of *E. coli* K-12 were isolated from various organs in the mice and assessed for relative survival. Significant dose-related reductions in levels of the repair-deficient relative to the repair-proficient strain, indicative of

genotoxicity, were observed among the bacteria recovered from the liver, lung, spleen, kidney, stomach and intestines over all doses tested.

Sasaki *et al.* (1997a) measured DNA damage using the alkaline single-cell gel electrophoresis (Comet) assay in male CD-1 mice following a single oral dose by gavage at a dose of 100 mg MX/kg body weight. Following administration mice were sacrificed at 0, 1, 3, 6 or 24 hours after treatment. Tissue samples from the liver, lung, kidney, brain, spleen and bone marrow as well as mucosa from the stomach, jejunum, ileum, colon and bladder were assayed for strand breaks and alkali-labile sites. DNA alkaline-elution migration patterns indicative of apoptotic cells were not scored. MX induced statistically significant increases in DNA damage in the liver, kidney, lung, and brain, and in all mucosal samples. DNA damage persisted in the gastrointestinal and urinary tracts for 6 to 24 hours after dosing. However, DNA damage in the liver peaked at one hour after exposure and returned to control levels by three hours. MX did not appear to cause DNA damage in the bone marrow of mice.

Nunn *et al.* (1997) examined unscheduled DNA synthesis in mouse hepatocytes from mice treated with a single oral dose of 100 mg/kg. Hepatocytes were collected from mice three or 16 hours following administration of MX. An increased net nuclear grain count was observed three hours after administration of MX, which usually is considered a positive finding. However, the authors did not conclude that this increase represented a positive finding since the effect was only marginally positive and the increase was not observed 16 hours after dosing. The results are not surprising in light of observations by Sasaki *et al.* (1997a) that liver DNA damage was high one hour after dosing but had returned to background levels three hours after exposure.

Holme *et al.* (1999) treated $B6C3F_1$ mice with i.p. injections or intra-rectal intubations at doses of 40 or 80 mg MX/kg body weight. Some mice were pretreated with DNA repair enzyme inhibitors, cytosine-1-β-D-arabinofuranoside (AraC) and hydroxyurea (HU), at doses of 200 mg/kg body weight 30 minutes prior to MX administration. One hour after exposure to MX, various organs were harvested and analyzed for single strand breaks using alkaline elution methods. Dose-related DNA damage in the liver, kidney, spleen and colon was observed in mice pretreated with DNA repair enzyme inhibitors, but not in mice without pretreatment.

Genotoxicity in non-bacterial, non-mammalian species

The DNA-damaging potential of MX also has been studied in fish, shellfish and plants. MX induced single strand breaks and alkali-labile sites, as measured by an alkaline elution assay, in trout erythrocytes and hepatocytes (Hodges *et al.*, 1997; Mitchelmore and Chipman, 1998). Exposure of two species of shellfish (*Patunopecten yessoensis* and *Tapes japonica*) to artificial sea water containing MX at concentrations ranging from 0.25 to 5.0 ppm for four hours resulted in dose-related increases in gill DNA damage, as measured by the alkaline single-cell gel electrophoresis assay (Sasaki *et al.*, 1997b). DNA strand breaks were also observed in the digestive glands of mussels (*Mytilus*

edulis 1) treated with MX (Mitchelmore *et al.*, 1998). Plants (*Tradescantia*) treated with doses of MX, either through absorption by plant cuttings or by direct application to the reproductive parts of the plant, caused increases in micronuclei in pollen mother cells (Helma *et al.*, 1995).

DNA adducts

Several studies have observed MX-DNA adduct formation *in vitro* (Le Curieux *et al.*, 1997; Meier *et al.*, 1989; Munter *et al.*, 1998; 1999; Franzen *et al.*, 1998b; Schut *et al.*, 1991), while others have not (Alhonen-Raatesalmi and Hemminki, 1991). One study reported preliminary findings of the formation of MX-DNA adducts *in vivo* (Schut *et al.*, 1991). At least five different adducts were observed among these reports, and it is not clear if there are any common observations among the studies. These findings may indicate that MX induces different types of DNA adducts under different reaction conditions. Indeed, MX exists in its closed ring form in acidic media, and is mostly in its open-ring form (z-MX) at physiological pH, which in turn is in equilibrium with its isomeric form EMX (Figure 2) (Meier *et al*., 1987a). Alternately, these observations may indicate, as Tuppurainen (1997) has proposed, that the mechanism of DNA damage of MX is not a result of site-specific DNA adduction, but rather is purely a thermodynamic phenomenon (see section below entitled Theoretical Studies of Reactivity and Section 3.4 Mechanism). The reports examining DNA adducts of MX are briefly described below.

Meier *et al.* (1989) reported in an abstract that they had measured DNA adducts in *Salmonella typhimurium* TA100, rat hepatocytes and rat embryonic cell lines treated with MX (reaction conditions not reported). DNA adducts were analyzed by ^{32}P-postlabeling. A dose-dependent increase in DNA adduct formation was observed for all three cell lines. In all three cases only one major adduct was detected. It is not clear from the report whether this was the same adduct in each case. The structure of the DNA adduct observed in each cell line was not characterized. DNA-adduct levels were about two per 10^7 DNA bases at a dose of ten μg MX/mL in the two mammalian cell lines. A comparable adduct level was observed in *Salmonella* treated with a dose of one μg MX/mL.

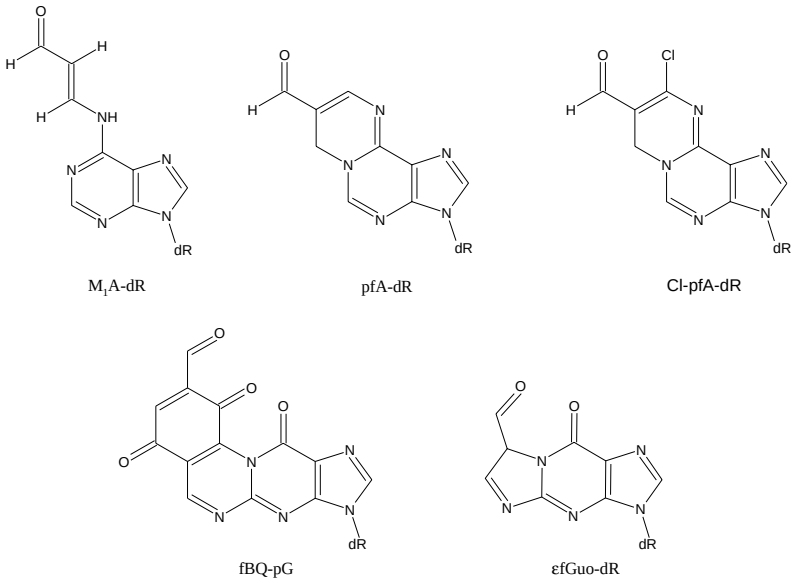
Alhonen-Raatesalmi and Hemminki (1991) reacted MX with the four deoxyribonucleosides and a dinucleotide for at least 21 hours at pH 7.4 and 37°C. No stable adducts were observed by high performance liquid chromatography. A minor amount of adenine was noticed as a depurination product.

Schut *et al.* (1991) reported in an abstract observing two DNA adducts following reaction of MX with calf thymus DNA for 18 hours. The two adducts were not characterized. The report described preliminary evidence that the two adducts were also present *in vivo* (tissue site not reported) following oral administration (dose not reported) of male $B6C3F_1$ mice with MX.

Other groups have produced and characterized MX-derived DNA adducts *in vitro*. Le Curieux *et al.* (1997) reacted MX with deoxyadenosine in buffered solutions at pH 4.6, 6.0 or 7.4. They observed the formation of one adduct, whose structure was characterized as 3-(2'-deoxy-β-D-ribofuranosyl)-N^6-adenosinyl)propenal (M_1A-dR) (Figure 3). The adduct was obtained at all the pH reaction conditions tested. The investigators also observed the M_1A-dR adduct in calf thymus DNA that was reacted with MX for four days at pH 6.5. In subsequent work by this laboratory, Munter *et al.* (1998) reacted MX with deoxyadenosine for eight days at pH 7.4 and reported the formation of two additional adducts. The structures were characterized by nuclear magnetic resonance spectroscopy and mass spectrometry. These adducts were comprised of a propeno bridge between the N-1 and N^6 positions of adenine. The products were 3-(2'-deoxy-β-D-ribofuranosyl)-7*H*-8-formyl[2,1-*i*]pyrimidopurine (pfA-dR) and 3-(2'-deoxy-β-D-ribofuranosyl)-7*H*-8-formyl-9-chloro[2,1-*i*]pyrimidopurine (Cl-pfA-dR) (Figure 3). In reaction of MX with calf thymus DNA at pH 7.4 for four days, only the pfA-dR adduct was detected (Munter *et al.*, 1998).

Based on electronic and structural properties and from site-specific mutations in *Salmonella typhimurium* TA100 and TA98, several groups of investigators have proposed that the most favorable interaction of MX and DNA would occur with guanine (Tuppurainen and Lotjonen, 1993; DeMarini *et al.*, 1995; Knasmüller *et al.*, 1996).

Figure 3. DNA adducts of MX



Franzen *et al.* (1998b) reacted MX with adenosine, guanosine, and cytidine in aqueous solutions at pH 7.4 for ten days. HPLC analyses showed that a clearly detectable adduct was formed in the reaction with guanosine. This product was isolated and identified as 10-formyl-1, N2-benzoquinone propenoguanosine (fBQ-pG) (Figure 3). fBQ-pG is the product a guanosine and two molecules of MX. The study authors described a

mechanism of formation involving sequential reaction of two molecules of the open-ring form of MX (z-MX).

Recently, Munter *et al.* (1999) reacted MX with guanosine, cytidine, thymidine, and calf thymus DNA in aqueous solutions. In the reactions of MX with cytidine or thymidine, no base-modified nucleosides were detected. HPLC analyses of the reaction mixture of MX with guanosine showed that one adduct was formed. The product was identified as 3-(β-D-ribofuranosyl)-7-formylimidazo[1,2-a]purin-9(4H)-one (εfGuo-dR) (Figure 3). The εfGuo-dR adduct was not observed in the hydrolysate of the calf thymus DNA reacted with MX, at a detection limit of five adducts per 10^7 bases. Since MX causes mutations, which appear to arise preferentially from lesions at guanosine residues as observed in *Salmonella typhimurium* TA100, TA98 and mammalian cells (Tuppurainen and Lotjonen, 1993; DeMarini *et al.*, 1995; Tuppurainen, 1997), the lack of detection of the εfGuo-dR adduct in calf thymus DNA suggests that DNA lesions at guanine bases may occur by some mechanism other than formation of stable DNA adducts with guanine.

Theoretical studies of reactivity

Several investigators have studied the structural and electronic properties of MX and related compounds to try to understand why MX is such a potent mutagen in bacteria (LaLonde *et al.*, 1992; Tuppurainen *et al.*, 1991; 1992, Tuppurainen and Lotjonen, 1993). Using data on MX and 16 structurally related compounds, significant correlations were found between mutagenicity (in *Salmonella typhimurium* strain TA100) and several electronic parameters of chlorofuranones (Tuppurainen *et al.*, 1992). These parameters included the energy of the lowest unoccupied molecular orbital (LUMO) (r = 0.9607), electron affinity (r = 0.9557), LUMO electron density at the carbon in the 3rd position (r = 0.8855), and partial charge of the number 3-carbon (r = 0.8812). The slope between mutagenicity and LUMO energy is exceptionally high compared to estimates for other classes of mutagenic compounds (Tuppurainen, 1997). These correlations suggest that the high reductive potential of MX is closely related to its mutagenicity. Tuppurainen (1997) hypothesized that MX pulls an electron from DNA following non-covalent interaction with DNA, resulting in an ionized DNA base (e.g., $CG^{+\bullet}$). This would presumably result in an abasic site or other DNA lesion that could be misrepaired. Tuppurainen (1997) noted that this hypothesis is consistent with energy calculations which indicate that the mutational hot spot in *Salmonella typhimurium* TA100 represents a global minimum energy position for a positive "hole" in ionized DNA. Thus, it appears likely that MX induces DNA damage through a thermodynamic mechanism, rather than (or in addition to) DNA adduction.

3.3.2 Tumor Initiation and Promotion

Owing to its significant mutagenic potential and its ability to induce tumors at multiple sites in rats, MX is likely to act as a tumor initiator in classic initiation/promotion assays, although MX has not been tested as an initiator in such assays. MX has produced mixed results as a tumor promoter in two rodent studies (Nishikawa *et al.*, 1999; Steffensen *et*

al., 1999). Steffensen *et al.* (1999) in separate experiments treated male F344 rats and Balb/cA mice with either MX via drinking water or intra-rectally, following treatment with the colon carcinogens 1,2-dimethylhydrazine or azoxymethane. MX treatment orally did not increase aberrant crypt foci or increase the number or size of tumors in the colon or rectum of initiated animals, but MX treatment intra-rectally apparently promoted growth of aberrant crypt foci in initiated rats and mice. Nishikawa *et al.* (1999) fed male Wistar rats (30 animals per group) diets containing 100 ppm N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for eight weeks. Initiation with MNNG was followed by administration of MX in the drinking water at concentrations of 0, 10 or 30 ppm for 57 weeks (promotion). All surviving animals were sacrificed at week 65 of the study. Treatment with MX enhanced the incidence and multiplicity of adenocarcinoma of the glandular stomach and the incidence of atypical hyperplasia of the glandular stomach compared to rats given MNNG alone. Promotion with MX also increased the incidences of thyroid follicular cell hyperplasia and combined incidences of cholangiocarcinomas and cholangiomas in MNNG-induced animals, but the increases were not statistically significant.

3.3.3 Cellular Proliferation

The tumor-promotion findings of Nishikawa *et al.* (1999) are consistent with observations that MX induced cellular proliferation in the gastric mucosa of rats (Furihata *et al.*, 1992; Nishikawa *et al.*, 1994). Four-week old male Wistar rats were given MX via drinking water at concentrations of 0, 6.25, 12.5, 25 and 50 ppm for five weeks (Nishikawa *et al.*, 1994). The authors reported a dose-related increase across the control and lowest three dose groups in the cell proliferation rate (measured by BrdU-labeling) in the mucosal epithelia of the gastric fundus and in levels of urinary lipid peroxidation. Cell proliferation and peroxidation levels in the 50 ppm group were lower than the expected trend, which the authors attributed to toxicity as gastric erosion was noted in the highest dose groups. Furihata *et al.* (1992) administered to male F344 rats a single gavage doses of 20 to 48 mg MX/kg body weight and reported an increase in cell proliferation of the glandular stomach, as measured by the induction of replicative DNA synthesis and ornithine decarboxylase. No studies of MX-induced cellular proliferation in tissues other than the gastrointestinal tract were located.

3.3.4 Structure-Activity Comparisons

Chlorinated and brominated furanones, structurally similar to MX, were observed to be mutagenic in bacteria. Fawell and Horth (1990) describe studies in which brominated analogues of MX were synthesized and tested for mutagenicity in *Salmonella typhimurium* strains TA100 and TA98. 3-Bromo-4-(dibromomethyl)-5-hydroxy-2(*5H*)-furanone, 3-bromo-4-(dichloromethyl)-5-hydroxy-2(*5H*)-furanone, and 3-chloro-4-(dibromomethyl)-5-hydroxy-2(*5H*)-furanone were all mutagenic. The fully brominated analogue of MX was equivalent in mutagenic potency to MX. Brominated analogues of MX have also been shown to be mutagenic by others (Suzuki and Nakanishi, 1995).

3-Chloro-4-(chloromethyl)-5-hydroxy-2(*5H*)-furanone, which differs from MX in that it contains a chloromethyl group instead of a dichloromethyl group, was mutagenic in *Salmonella typhimurium* strains TA1535, TA100, TA102, TA98 and TA97, and caused mutations in the HPRT locus of Chinese hamster ovary cells (Niittykoski *et al.*, 1995). 3-Chloro-4-(chloromethyl)-5-hydroxy-2(*5H*)-furanone was a less potent mutagen than MX.

LaLonde *et al.* (1991a) studied the mutagenicity of MX and eleven structurally similar analogues in *Salmonella typhimurium* strain TA100. Of these compounds, only MX has been tested for carcinogenicity. The compounds studied were:

1) 3-chloro-4-(dichloromethyl)-5-hydroxy-2(*5H*)-furanone (MX)
2) 3-chloro-4-(dichloromethyl)-2(*5H*)-furanone
3) 3-chloro-4-(chloromethyl)-5-hydroxy-2(*5H*)-furanone
4) 3-chloro-4-(chloromethyl)-2(*5H*)-furanone
5) 4-(chloromethyl)-5-hydroxy-2(*5H*)-furanone
6) 4-(chloromethyl)-2(*5H*)-furanone
7) 3-chloro-4-methyl-5-hydroxy-2(*5H*)-furanone
8) 3-choro-4-methyl-2(*5H*)-furanone
9) 4-methyl-5-hydroxy-2(*5H*)-furanone
10) 4-methyl-2(*5H*)-furanone
11) 4-(dichloromethyl)-5-hydroxy-2(*5H*)-furanone
12) 4-(dichloromethyl)-2(*5H*)-furanone

Compounds 1 through 7 were mutagenic, while compounds 8 through 10 were not. Compounds 11 and 12 were unstable. Compound 2 was equal in mutagenic potential to MX (compound 1), and these two compounds were the most mutagenic of the set. In comparing the mutagenicity of compounds 3 through 7 to MX, the authors observed that each replacement of a chlorine with hydrogen resulted in a reduction of the mutagenic potential by 10- to 1000-fold. LaLonde *et al.* (1992) compared the chemical and electronic properties of these structurally similar chlorinated furanones with their mutagenic activities in *Salmonella typhimurium* TA100 as published in earlier reports from their laboratory. Two general findings were made. First, as the LUMO energy decreased, mutagenicity increased. Second, the chlorinated furanones exhibited differing abilities to accept and stabilize an electron. As the stability of the resulting radical anion increased, so did its ability to induce mutations.

3.3.5 Pharmacokinetics and Metabolism

Studies of the pharmacokinetics of MX are limited. Ringhand *et al.* (1989) administered radiolabeled MX to six male Fisher 344 rats by oral gavage at a dose of approximately 40 mg/kg body weight. MX was dissolved in water adjusted to pH 8. Urine and fecal samples were collected 8, 24, 32 and 48 hours following exposure. Most of the MX dose (47 percent) was recovered in the feces. In the urine, 33.5 percent of the dose was recovered after 48 hours, most of which was recovered during the first 24 hours. Less than one percent was recovered in exhaled air. After 48 hours, some tissues retained a small proportion of the radiolabel: gastrointestinal contents (2.3 percent), blood (1.2

percent), liver (1.2 percent), muscle (1.6 percent), and several other tissues (each <1 percent).

Komulainen *et al.* (1992) studied the pharmacokinetics of MX in Han:Wistar rats following a single intravenous or gavage dose of radiolabeled MX (one to two mg/kg body weight). Approximately 20 to 35 percent of the radiolabel was excreted in the urine, and about 50 percent was recovered in the feces. The mean half-life of MX was 3.8 hours, but the elimination was multi- or bi-phasic with a long terminal half life, as traces of radioactivity could be measured in the blood for several days following exposure. The tissues lining the kidneys, stomach, small intestine and urinary bladder contained the highest levels of radioactivity. Lower levels of radioactivity were observed in the liver, lungs and esophagus.

Clark and Chipman (1995) studied the intestinal absorption of MX using an *in vitro* gut sac system. Transport of MX or MX-derived mutagens from the mucosal to the serosal compartments was measured using the standard reverse mutation assay in *Salmonella typhimurium* TA100. However, pre-incubation of MX with glutathione (1.0 mM) resulted in no detectable absorption of mutagens.

OEHHA was unable to locate any *in vivo* studies of MX metabolism in the scientific literature, except two studies which examined enzyme changes in various tissues of rats following exposure to MX (Heiskanen *et al.*, 1995; Meier *et al.*, 1996). MX has been shown *in vitro* to readily conjugate with glutathione, either directly or enzymatically (reviewed in Meier *et al.*, 1996). MX appears to be almost completely metabolized or converted *in vivo*, since only traces of MX were measured in the urine of rats, even at lethal doses (Komulainen *et al.*, 1994). However, the metabolites of MX are largely unknown.

Meier *et al.* (1996) investigated the changes in metabolizing enzymes in male rats treated by oral gavage with 0, 8, 16, 32 or 64 mg MX/kg body weight for 14 days. Urine and blood collected from rats in each dose group were analyzed for genotoxicity. Increased mutagenicity in *Salmonella typhimurium* TA100 was observed in the urine from high-dose animals only (64 mg/kg) relative to controls. This observation lead the authors to suggest that metabolic saturation of MX occurs between 32 and 64 mg/kg body weight in rats. Only animals from the high dose group were assayed for enzymatic changes. Significant reductions in liver catalase, cytochrome P450 reductase, aminopyrine demethylase, and aryl hydrocarbon hydroxylase (AHH) activity and an increase in liver urate oxidase were observed in MX-treated rats relative to controls. No significant changes were observed in liver fatty acyl CoA oxidase, glutamylcysteine synthetase, glutathione-S-transferase (GST), or glutathione peroxidase activity. The authors concluded that the findings indicate that MX is extensively detoxified *in vivo*; however, they also noted that the mechanism of these metabolic changes and their relevance to *in vivo* metabolism requires further investigation.

Heiskanen *et al.* (1995) investigated the effect of MX exposure of rats on metabolizing enzymes in the liver, kidney, duodenum and lung. Groups of Wistar rats (15/sex/dose) were administered MX by gavage at doses of 0 (vehicle control) or 30 mg/kg body weight (low dose groups) for 18 weeks. Additional groups of male and female rats were administered MX at 45 mg/kg for seven weeks which was increased to 60 mg/kg for two weeks and further increased to 75 mg/kg body weight for five weeks (high dose groups). For both males and females, dose-related decreases in 7-ethoxyresorufin-O-deethylase (EROD) activity was observed in the kidney and liver, but not in the duodenum and lung microsomal preparations. Similarly, a dose-related decrease in pentoxyresorufin-O-dealkylase (PROD) activity was observed in the kidney of female mice. With respect to conjugating enzymes, a dose-related increase in UDPG-glucuronosyltransferase (UDPGT) activity was observed in the kidneys of MX-treated rats, but not in the liver, duodenum or lung. Similarly, a dose-related increase in GST activity was observed in the kidney, liver and duodenum of MX-treated female rats.

Thus, it appears that MX inhibits cytochrome P450, possibly through suicide inhibition, which is essentially an inhibition of a detoxification pathway since MX is a direct-acting mutagen. Inhibition of oxidative metabolic enzymes would put a greater demand on other detoxification pathways such as glutathione or glucuronide conjugation.

3.3.6 Pathology

Komulainen *et al.* (1997) described the pathological observations and histopathological characterizations of the tumors in the MX-treated rats. The observations for the organ systems primarily affected by MX are discussed below.

Liver: The authors noted that the livers of MX-treated rats exhibited sharply demarcated nodules on the surface, and most of these lesions contained a clear liquid. The livers also appear to be mottled. Increased liver to body weight ratios were increased for the mid- and high-dose females and the high-dose males relative to controls. Liver adenomas are generally believed to progress to carcinomas (McConnell *et al.*, 1986). For this reason, combined incidences of liver adenoma and carcinoma among male and female rats were reported (Tables 1 and 2). Similarly, in female rats the incidences of cholangioma and cholangiocarcinoma (tumors of the epithelia of the bile ducts) were combined.

Thyroid: The thyroid gland showed the most dramatic tumorigenic response for both male and female rats (Komulainen *et al.*, 1997). The authors noted enlargement of the thyroid glands in “several” of the mid- and high-dose rats of both sexes, based on a visual observation at necropsy; the thyroid glands were not weighed (Komulainen *et al.*, 1997; Komulainen, 2000). Komulainen *et al.* (1997) stated that the follicular adenomas preceded carcinomas. Komulainen *et al.* (2000) provided combined incidence of follicular cell adenoma and carcinoma cancers. Combined tumor incidences reached nearly 100 percent in the high dose groups. Komulainen *et al.* (1997) measured the mean plasma levels of the thyroid hormones, thyroid stimulating hormone (TSH), T_4 and T_3, at the end of the study in MX-treated animals and found that they were not different from

levels in the control rats. These observations strongly suggest that thyroid cancers induced by MX are not due to a proliferative mechanism resulting from thyroid hormone disruption (see Section 3.4 Mechanism).

Mammary gland: No pathological details on the mammary tumors in female rats were provided by the study authors (Komulainen *et al.*, 1997). However, mammary gland carcinoma may arise from atypic hyperplasia and adenoma, and thus may be combined if the data warrant doing so (McConnell *et al.*, 1986). Combined incidence of mammary gland adenoma and carcinoma as well as mammary gland atypic hyperplasia, adenoma and carcinoma were tallied (Table 2).

Other sites: No pathological details were provided by the study authors for tumors of the hematopoietic system, skin, lung, pancreas and adrenal gland (Komulainen *et al.*, 1997). It is generally believed that Langerhan's cell adenoma of the pancreas is a precursor to carcinoma (McConnell *et al.*, 1986), thus combined incidence of these pancreatic cancers was reported for male rats (Komulainen *et al.*, 2000).

3.4 Mechanism

MX is genotoxic, causing a wide range of DNA damage *in vitro* and *in vivo* (Tables 3 and 4). MX is a direct acting mutagen and clastogen. Several DNA adducts have been characterized. Theoretical studies of MX's reactivity predict that MX's high mutagenic potential in bacteria is related to its high reductive potential. A genotoxic mode of action is likely responsible for the observed carcinogenic effects.

The specific mechanism by which MX induces DNA damage is not known, but the available evidence suggests the possibility of two distinct mechanisms. First, DNA adducts of MX have been observed with adenine and guanine; several structures have been characterized (Figure 3). DNA adducts or adduct-derived abasic sites could be misrepaired, leading to mutations or strand breaks. The mutational hot spot in *Salmonella typhimurium* TA 100 suggests that MX preferentially attacks a guanine residue, which is misrepaired resulting in the predominant GC $\rightarrow$ TA transversions. However, the inability to measure MX-guanine adducts upon reaction of MX with intact (calf thymus) DNA suggests that stable DNA-adduct formation may not be the predominant means of altering guanine bases.

A separate mechanism of MX-induced genotoxicity has been proposed by Tuppurainen (1997). Based on comparisons of mutagenic potential and the electronic properties of MX and related chlorinated furanones, Tuppurainen and other researchers (LaLonde *et al.*, 1992; Tuppurainen *et al.*, 1991; 1992, Tuppurainen and Lotjonen, 1993) have observed correlations between the reductive potential of these compounds and their mutagenicity (see above). Tuppurainen (1997) hypothesized that, as a result of non-covalent interaction, MX pulls an electron from DNA, resulting in an ionized DNA base (e.g., $CG^{+\bullet}$). This would presumably result in an abasic site or other DNA lesion that could be misrepaired. Tuppurainen (1997) noted that energy calculations indicate that the

mutational hot spot in *Salmonella typhimurium* TA100 represents a global minimum energy position to stabilize the ionized DNA. Thus, it appears likely that MX induces DNA damage through an unusual thermodynamic mechanism, rather than (or in addition to) DNA adduction.

The strongest tumorigenic response with both male and female rats was in the thyroid gland. It has been well documented that rats are susceptible to the proliferative effects of sustained disruption of thyroid hormone balance, resulting in hyperplasia and neoplasia (U.S. EPA, 1998b). Thus, it is important to investigate the propensity of MX to induce cell proliferation in the thyroid. As noted above, the authors did report "several" of the rats in the mid- and high-dose groups of both sexes had enlarged glands (visual inspection at necropsy), although no measurements of the thyroid gland weights were made (Komulainen, 2000). Also, MX has been reported to cause increases in cellular proliferation in other tissues (gastric mucosa) (Nishikawa *et al.*, 1994). It remains unclear whether the high rate of thyroid neoplasia can account for the macroscopic findings of enlarged glands or whether MX-induces additional proliferative events in the thyroid. However, it does not appear that MX causes thyroid tumors by disrupting thyroid hormone balance. Komulainen *et al.* (1997) measured the mean plasma levels of the thyroid hormones TSH, T_4 and T_3 at the end of the study in MX-treated animals and found that they were not different from levels in the control rats. Komulainen *et al.* (1998) also conducted a short-term studies in which Wistar rats (six/dose/sex) were administered MX by oral gavage at daily doses of 1, 10 or 60 mg/kg body weight for 7 or 21 days. MX did not affect TSH or T_4 levels in the blood of either sex. A slight increase (25%) in the blood concentration of T_3 was observed in male rats treated with 60 mg/kg after 7 days of dosing but not after 21 days. These findings suggest that TSH promotion (i.e., sustained disruption of thyroid hormone balance) is not likely to play a significant role in MX-induced follicular cell tumors of the thyroid in rats.

The mechanism of carcinogenesis for MX at other tissue sites (Tables 1 and 2) is unknown, although DNA damage has been measured in the liver and lung of rodents treated with MX (Table 4), consistent with a genotoxic mode of action.

4 SUMMARY AND CONCLUSIONS

4.1 Summary of Evidence

Treatment of male or female rats with MX via drinking water for two years resulted in increased rates of tumor formation at multiple sites in both sexes (Komulainen *et al.*, 1997; 2000). Statistically significant increases in the incidences of liver adenoma and carcinoma (combined), adrenal gland cortical adenoma, thyroid follicular cell adenoma, and thyroid follicular cell carcinoma were observed for both male and female MX-treated rats relative to controls. Significant dose-related trends in these tumors were also observed. Increased incidences of mammary gland adenocarcinoma, mammary gland

fibroadenoma, and liver cholangioma also were observed among MX-treated female rats relative to controls. The thyroid gland was the most sensitive tissue for both sexes, reaching nearly 100 percent tumor incidence in the high dose groups of males and females. In addition, significant dose-related trends were observed for tumors of the skin and lung in male rats, and the hematopoietic system of female rats. MX has not been tested for carcinogenic activity in mice.

MX is a direct acting mutagen and clastogen. MX caused mutations in over 35 strains of bacteria. MX is one of the most potent mutagens ever tested in the reverse mutation assay in *Salmonella typhimurium* TA100, in the forward mutation assay in *Salmonella typhimurium* TM677, or the lamba prophage Microscreen assay. MX caused mutations in mouse lymphoma cells and Chinese hamster ovary cells. MX induced chromosomal aberrations and SCEs in Chinese hamster ovary cells, rat lymphocytes, and mouse lymphoma cells. MX caused increases in DNA damage, as measured by various alkaline elution techniques, in human lymphoblastoid cell lines, human peripheral blood mononuclear cells, human-derived HL-60 cells, human kidney epithelial cells, pig kidney cells, rat hepatocytes, rat testicular cells, Chinese hamster ovary cells, and trout erythrocytes. MX induced unscheduled DNA synthesis in rat and mouse hepatocytes. *In vivo* genotoxicity studies of MX were generally positive for indicators of DNA damage in a variety of tissues, with the exception of bone marrow and marrow-derived cells. Several studies observed increases in single strand breaks and alkali-labile sites, micronuclei or SCEs in blood lymphocytes, kidney, stomach, jejunum, ileum, colon, duodenum, liver, lung, brain, spleen or bladder of rodents given MX via oral or i.p. administration.

In classic initiation/promotion studies, MX was reported to act as a tumor promoter, although these findings are mixed, and MX has not been tested as an initiator. Chlorinated and brominated furanones, chemicals structurally similar to MX, also induced mutations in bacterial test systems.

Data on the metabolism of MX are lacking. Data on the pharmacokinetics of MX are limited. Following ingestion, MX is absorbed to a moderate degree (about 40 percent in rats) and is distributed to many tissues. MX has been shown to inhibit some oxidative liver enzymes such as cytochrome P450s, and also appears to increase the activity of some conjugating enzymes such as glucuronosyltransferases.

Although the precise carcinogenic mechanism is not known, MX likely induces tumors through a genotoxic mode of action. MX may induce mutations through a couple of possible mechanisms. One possibility is that MX directly binds to DNA (several DNA adducts have been characterized), which could be misrepaired. Additionally there is compelling evidence to suggest that MX induces mutations through a non-covalent, thermodynamic mechanism in which MX transfers an electron from DNA resulting in an ionized DNA base (see Section 3.4 Mechanism).

In male and female rats, the thyroid gland and liver were the primary target organs (Komulainen *et al.*, 1997). The mechanism of MX-induced thyroid tumors is not known at this time, although available evidence suggests that MX does not cause thyroid tumors by a mechanism of sustained thyroid-hormone disruption.

4.2 Conclusion

MX induced dose-related increases in tumor incidences at multiple sites in both male and female rats relative to controls (Komulainen *et al.*, 1997; 2000). MX has not been tested in mice. The carcinogenic weight of evidence is supported by extensive observations of mutagenicity and clastogenicity in test systems *in vitro* and in rats and mice *in vivo*.

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