Abstract

DANIEL T. SHAUGHNESSY. Mutation Spectra of the Drinking Water Mutagen 3-Chloro-4-Methyl-5-Hydroxy-2(5H)-Furanone: Comparison to MX. (Under the Direction of Dr. DAVID DEMARINI)

Disinfection of drinking water by chlorination produces a number of mutagenic by-products. The chlorinated drinking water mutagen 3-chloro-4-methyl-5-hydroxy-2(5H)-furanone (MCF) occurs at concentrations similar to or greater than that of the related furanone 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX). MCF and MX differ structurally only by replacement of a 4-methyl in MCF with a 4-dichloromethyl in MX; yet, MCF is significantly less mutagenic than MX and produces different adducts when reacted with nucleosides or DNA. To explore further the effects that these structural differences might have on the biological activity of MCF and MX, we determined the mutation spectra of MCF in *Salmonella* strains TA100 and TA104 and of MX in strain TA104. Our results show that the replacement of the 4-methyl in MCF with a 4-dichloromethyl group in MX not only dramatically shifts the mutagenic potency but also shifts the mutagenic specificity from an almost equal targeting of GC and AT sites by MCF to an almost exclusive targeting of GC sites by MX.

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Introduction

Rationale

Disinfection of drinking water by chlorination is one of the most important public health advances of the 20th century. Among the diseases controlled by the disinfection process are typhoid fever, cholera, amoebic dysentery, shigellosis, *Cambylobacter* Enteritis, giardiasis, schistosomiasis, and several viruses, notably hepatitis A, Norwalk, and rotaviruses (IARC, 1991). However, the occurrence of a variety of mutagenic and carcinogenic by-products formed by disinfection with chlorine poses a dilemma: the production of microbiologically safe drinking water must be balanced with minimizing possible health risks from disinfection by-products. Among the mutagenic products formed in the chlorination of drinking water are trihalomethanes, halogenated acetic acids, halogenated acetonitriles, chlorinated ketones, halogenated phenols, and chlorinated furanones (IARC, 1991). A significant difference has been found between consumers of chlorinated drinking water (based on population-based estimates of exposure to chlorinated water from municipal waterworks) and consumers of nonchlorinated water (groundwater from private wells), with consumers of chlorinated water having a relative risk of 1.2 for bladder cancer and 1.2-1.4 for kidney cancer (Koivusalo et al., 1994). Much of the mutagenic activity of chlorinated drinking water can be attributed to chlorinated organics found in the acidic semivolatile and nonvolatile fractions eluted from XAD macroreticular resins (IARC, 1991). Among these

chlorinated organics are 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) and the structurally related mutagen 3-chloro-4-methyl-5-hydroxy-2(5H)-furanone (MCF). MX is one of the most potent mutagens known, producing more than 5000-6000 revertants/nanomole in *Salmonella* TA100 (Franzen et al., 1998; Tikkanen and Kronberg, 1990). MCF is significantly less potent than MX, producing only 0.3 revertants/ μ g in *Salmonella* TA100 (Franzen et al. 1998). Concentrations of both MX and MCF in chlorinated drinking water are similar; estimates from several countries have been in the range of 3 to 67 ng/L, the highest concentrations being found in Finnish drinking water samples (Smeds et al., 1997). MX and MCF have been shown to differ widely in reaction specificity with particular nucleosides and calf thymus DNA (Munter et al., 1996, 1998, 1999; Le Curieux et al., 1997). In the present study, we have determined the mutation spectra of MCF in relation to MX to determine if the spectra of these two compounds can explain the differences in mutational specificity reported in nucleoside adduct studies of these compounds. We have determined the mutation spectra of MX and MCF in *Salmonella* TA104 and of MCF in TA100 using colony probe hybridization techniques, PCR amplification, and DNA sequencing. The results were interpreted in terms of structure-activity relationships that may explain both the differences in mutational specificity and the dramatic difference in mutational potency between the two compounds.

Drinking Water Disinfection by Chlorination

Disinfection using chlorine (in one form or another) is one of the most common methods used to produce microbiologically safe drinking water

(IARC, 1991). When modern water treatment practices are followed, including prechlorination, flocculation, coagulation, sedimentation, and filtration followed by terminal disinfection, pathogenic bacteria and viruses essentially can be completely removed or inactivated (White, 1999a). Pretreatment is often necessary for surface waters, which are generally more prone to contamination than groundwater. Storage of water in a reservoir can reduce the number of pathogenic organisms in the raw water through sedimentation of suspended solids. Dissolved impurities not removed by settling are removed by coagulation. Aluminum or ferric salts are coagulants commonly used to create large particles called "flocs," which are removed by sedimentation followed by deep-bed filtration through sand (IARC, 1991). Although the primary objective of chlorination is disinfection, other purposes include control of tastes and odors, removal of certain organic colors by bleaching, prevention of algal growths and the control of slime growths (which are also caused by microorganisms), destruction of hydrogen sulfides, and removal of iron and manganese (White, 1999a). Among the different chlorine species, the most effective germicide is hypochlorous acid (HOCl), formed when chlorine gas is dissolved in water.

Hypochlorous acid is a weak acid that undergoes partial dissociation to produce a hypochlorite ion (OCl^-) and a hydrogen ion as follows:

The total concentration of hypochlorous acid, hypochlorite ion, and free chlorine is known as "free available chlorine" (IARC, 1991). A critical determinant in predicting the germicidal efficiency of a disinfectant is

CT factor, the product of chlorine disinfectant concentration and contact time. A large number of compounds present in raw water, including inorganic reducing agents and nitrogenous compounds, combine with free chlorine species to form combined chlorine species that are less effective disinfectants. Chlorine demand refers to the dosage of chlorine needed to produce a free chlorine residual in which HOCl accounts for 85 percent of the total available chlorine. CT values are established for the most resistant organisms that cause waterborne disease outbreaks, and currently the U.S. Environmental Protection Agency has developed CT values for the inactivation of Giardia cysts and enteric viruses (White, 1999a).

Ozone is an alternative disinfectant used in the treatment of drinking water, especially in Western Europe. A powerful oxidant, ozone is an effective germicide and is capable of rapidly oxidizing inorganic complexes (e.g., sulfates, ferrous iron, and nitrites) and degrading organic materials (including phenolic compounds). The ability of ozone to destroy taste-forming phenolic compounds makes ozonation an attractive alternative to chlorination. When bromide ions are absent from the source water, the primary by-products of disinfection with ozone are aldehydes and organic acids, which are easily biodegraded by microorganisms that may be reintroduced into the water at the filtration step after preozonation. Biological activated carbon (BAC) that is used, for example in a treatment plant in Laval, Quebec, is a wood-based carbon that has a porous structure favorable for bacterial colonization. Because ozone decays rapidly in water, it must be used in conjunction with a disinfectant that provides a protective residual in the water distribution system and also inactivates microorganisms released from the filter media in the biodegradation step

(White, 1999b).

Disinfection By-products from Chlorination

Genotoxic by-products, including trihalomethanes, halogenated acetic acids, halogenated phenols, and chlorinated furanones, are formed from reactions of aqueous chlorine with humic substances found in raw water. Humic substances found in natural surface waters are complex organic structures formed from the degradation of plant material. Lignin, a primary component of woody tissue, is a phenolic polymer thought to be a major precursor of humic substances (Christman et al., 1983). Christman et al. (1983) reported that chlorination of humic and fulvic acids derived from natural surface water produced more than 100 different reaction products, including trihalomethanes and tri- and dichloroacetic acids. Xu et al. (1997) have demonstrated the formation of MX from chlorination of organic fractions derived from river and lake water samples. Fulvic acids produced the highest amounts of MX among all fractions of the two samples. Oxidation and chemical degradation of fulvic and humic acids yield phenolic and aromatic compounds that are thought to be precursors to MX (Långvik et al., 1991; Norwood and Christman, 1987; Xu et al. 1997).

The most widely used method to concentrate drinking water samples for genotoxicity testing involves adsorption of organics from water on Amberlite XAD resins, followed by elution with organic solvents (Meier, 1988). During the preparation of drinking water extracts by this method, many of the volatile components (including trihalomethanes) are lost in solvent evaporation steps. Consequently, much of the reported mutagenicity of disinfection by-products in chlorinated drinking water

extracts refers to mutagenic nonvolatile organics (Meier, 1988).

Health Effects of MX

Among the acidic semivolatile and nonvolatile organics, MX is the most potent mutagen in a variety of in vitro and in vivo systems (DeMarini et al., 1995; Holme et al., 1999; Marsteinstredt et al., 1997). MX has been reported to cause a significant increase in sister-chromatid exchanges (SCEs) and chromosome aberrations in CHO cells (Meier et al., 1987; Mäki-Paakkanen et al., 1994), increased DNA single-strand breaks (SSBs) in human HL-60 cells (Marsteinstredt et al., 1997), and SSBs in human CCRF-CEM cells (Chang et al., 1991). Harrington-Brock et al. (1995) demonstrated a dose-dependent increase in mutant frequency in mouse lymphoma cells at the thymidine kinase locus for cells exposed to MX. The predominance of small colonies was consistent with observed clastogenicity, i.e., an increase in the number of chromatid breaks at higher MX doses. In vivo, MX induces nuclear anomalies in the gastrointestinal tracts of mice (Daniel et al., 1991), DNA damage in *E. coli* recovered from various organs of mice in the host-mediated assay (Fekadu et al., 1994), and DNA damage detected by the Comet assay in multiple organs of CD-1 mice exposed orally to MX (Sasaki et al., 1997). Komulainen et al. (1997) demonstrated that MX is a multi-site carcinogen in rats. In a recent study, Le Curieux et al. (1999) have demonstrated that both MCF and MX showed significant genotoxicity in the micronucleus assay (in mouse lymphoma cells) and in the unscheduled DNA synthesis assay in rat hepatocytes.

Contributions of MX and MCF to the Total Mutagenicity of Drinking Water

MX is one of the most potent mutagens detected in short-term bacterial mutagenicity tests, inducing 5000-6000 revertants/nanomole in *Salmonella* TA100 (Franzen et al., 1998; Tikkanen and Kronberg, 1990). Using gas chromatography/high-resolution mass spectrometry to analyze drinking water extracts from a variety of sources and disinfection treatment procedures, Smeds et al. (1997) found concentrations of MX ranging from 0.8 to 46 ng/L. Despite these low concentrations, estimates of the contribution of MX to the total mutagenicity (in *Salmonella* TA100) of Finnish, U.S., U.K., and Japanese extracts of drinking water range from 3 to 60% (Smeds et al., 1997).

A number of other halogenated hydroxy-furanones (in the closed form) or halogenated oxobutenoic acids (in the open form) with structural similarities to MX has been detected in chlorinated drinking water (Richardson, 1998) and studied for mutagenicity (Ishaguro, 1988; Tikkanen and Kronberg, 1990; Pekadu et al., 1994; Franzén et al., 1998). Among these is MCF, which has been found at concentrations similar to MX (Smeds et al., 1997; Kronberg and Franzén, 1993). However, MCF is significantly less potent than MX in several *Salmonella* strains. Based on a mutagenicity study in TA100, Franzén et al. (1998) observed that MX induced 5410 revertants/ μ g compared with 0.3 revertants/ μ g induced by MCF (or equivalently, 1176 revertants/nanomole induced by MX compared with 0.04 revertants/nanomole induced by MCF). MCF was, therefore, approximately 18000x less mutagenic than MX and accounts for less than 1% of the total mutagenicity of XAD extracts of chlorinated drinking water (Smeds et al., 1997).

Figure 1

Possible Adduct Formation by MX and MCF

MX and MCF differ in structure only by the substituent on C-4 of the furanone ring: MX has a 4-dichloromethyl group whereas MCF has a 4-methyl group (Figure 1). Several studies have compared the wide range of mutagenic potencies of MX and related halogenated hydroxy-furanones (LaLonde et al., 1997; Ishiguro et al., 1988; Tikkanen and Kronberg, 1990). A structural feature common to the most potent chlorohydroxyfuranones is the *cis* arrangement of the chlorine and dichloromethyl groups at the double bond (Ishiguro et al., 1988; Tikkanen and Kronberg, 1990). MX and MCF also differ in their reaction specificities with individual nucleosides, shown in Figure 2, which demonstrates the reactivity of MX and MCF with nucleosides and calf thymus-DNA (Hytinen and Jansson, 1995; LeCurieux et al, 1997; Munter et al. 1996, 1998, 1999). Experiments were performed in aqueous solution at physiological pH and temperature.

MX and MCF can exist in either a closed ring form or an open, ionized form, depending on pH conditions (Figure 1). In acidic aqueous conditions the closed ring form predominates, but in the pH range of 4-6 MX undergoes a tautomeric shift to the open, ionized form (Munter et al., 1999). In a recent study, Munter et al. (1999) reacted MX with guanosine, cytidine, thymidine, and calf thymus-DNA, and HPLC analysis revealed products only from reactions with guanosine. The product, efGuo (MX-G in Figure 2) was obtained at the highest yields at neutral or slightly alkaline pH conditions; very low yields were obtained at pH 4.6 and 6. ¹H NMR, ¹³C NMR, and high resolution mass spectrometry identified the product

Summary of known adducts formed by MX and MCF; superscript numbers next to the adduct abbreviations refer to the following references: ¹Hyttinen and Jansson (1995), ²Munter et al. (1999), ³Munter et al. (1998), ⁴Le Curieux et al. (1997), and ⁵Munter et al. (1996)

The names and abbreviations for the adducts are as follows: 3-(2'-deoxy- β -D-ribofuranosyl)-7-formylimidazo[1,2-a]purin-9(4*H*)-one (MX-G); [3-(2'-deoxy- β -D-ribofuranosyl)-7*H*-8-formyl-9-chloro[2,1-*i*]pyrimidopurine (MX-A1); [3-(2'-deoxy- β -D-ribofuranosyl)-7*H*-8-formyl[2,1-*i*]pyrimidopurine (MX-A2); 3-(2'-deoxyribofuranosyl-*N*⁶-adenosinyl)propenal (MX-A3); 5-(*N*⁶-adenosinyl)-3-chloro-4-methyl-2(5*H*)-furanone (MCF-A1); and 4-(*N*⁶-adenosinyl)-3-formyl-3-butenic acid (MCF-A2). Alternative names for MX-A3, MCF-A2, and MCF-A1 are as follows: (MX-A3) *N*⁶-(3-oxopropenyl)-dA, (MCF-A2) *N*⁶-(3-formyl-4-carboxy-2-butenyl)-dA, (MCF-A1) 5-[*N*⁶-dA]-3-chloro-4-methyl-2-furanone. Nomenclature for these adducts may vary in the literature.

Figure 2

Proposed Mechanism for the Formation of MX-G Adduct

Reaction with MX

Figure 3

as efGuo (3-(β -D-ribofuranosyl)-7-formylimidazo[1,2-a]purin-9(4H)-one). Figure 3 shows a proposed mechanism for the formation of efGuo (MX-G) in which the dichloromethyl group is initially attacked by the exocyclic amino group of guanosine, and HCL is displaced. An ethano bridge is formed by attack on the double bond of the butenoic acid structure by the endocyclic nitrogen. Chlorine atoms are substituted by hydroxyl groups, glyoxylic acid is cleaved, and the subsequent enol dehydrates to form MX-G. No G-adduct was detected in the reaction of the MX with calf thymus-DNA. In similar studies, reaction of MX with adenosine yielded the products MX-A2 and MX-A1. A possible mechanism for the formation of MX-A1 is shown in Figure 4. The mechanism by which the chlorine at C-9 is replaced by hydrogen to form MX-A2 is unknown. The initial step involves the transfer of a proton from the dichloromethyl group to the labeled carbon (Figure 4). The dichloromethyl group is attacked by the exocyclic amino group forming an enol intermediate. Displacement of the HCl, decarboxylation, and subsequent attack of the endocyclic nitrogen on the labeled carbon (MX had been previously labeled with ^{13}C) with yields the MX-A1 [3-(2'-deoxy- β -D-ribofuranosyl)-7H-8-formylpyrimido[2,1-i]purine]. LeCurieux et al. (1997) identified another product formed in the reaction of MX with adenosine, 3-(2'-deoxyribofuranosyl- N^6 -adenosinyl) propenal, referred to as MX-A3 in Figure 2. (An alternative name for this adduct is 3-[N^6 -deoxyadenosyl]-propenal).

Studies of possible adduct formation by MCF have also been conducted by Munter et al. (1996). No products were detected for reactions of MCF with guanosine or cytidine (Munter et al., 1996) or with calf thymus-DNA (LeCurieux et al., 1997). MCF-A2 (4-(N^6 -adenosinyl)-3-formyl-3-butenic

Proposed Mechanism for the Formation of the MX-A1 Adduct

MX Forms Propenoformyl Adducts with 2'-dAdo

Figure 4

Proposed Mechanism for the Formation of MCF-A1 and MCF-A2 Adducts

* = ^{13}C -labeled carbon

Figure 5

acid) was formed at higher yields at pH 6 than at pH 7.4, indicating that the initial step of the mechanism shown in Figure 5 is indeed an acid-catalyzed reaction. A proton transfer takes place from the methyl group to the labeled carbon. The exocyclic amino group of adenosine attacks the resulting methylene group with formation of an enol intermediate. Displacement of HCl and proton rearrangement yields MCF-A2 (Munter et al., 1996). MCF-A1 (5-(N⁶-adenosinyl)-3-chloro-4-methyl-5-hydroxy-2(5H)-furanone) was the only reaction product formed when the solvent was N,N-dimethylformamide (DMF). MCF-A1 may be formed by the initial attack by the exocyclic amino group on the aldehyde group of MCF (in the open form) yielding a gem aminohydrin that subsequently undergoes ring closure (Figure 5). The observed higher yield of MCF-A1 in aqueous solution at pH 7.4 may be due to the greater proportion of MCF in the open form.

Mutation Spectra of MX and MCF

Mutation spectra of MX and various related chlorinated furanones have been generated in several *Salmonella* strains with differing DNA repair backgrounds (Knasmüller et al., 1996; DeMarini et al., 1995; Hyttinen et al., 1995). In *Salmonella* strains bearing the *hisG46* allele (TA100 and TA1535), MX induced primarily GC → TA transversions and complex frameshifts (a frameshift with an adjacent base substitution) in the frameshift strain TA98. MX also induced primarily GC → TA transversions in a specially constructed strain of *Escherichia coli* (Watanabe-Akunama and Ohta, 1994) and at the *Hprt* gene in CHO cells as well (Hyttinen et al., 1996). MX, therefore appears to induce mutations primarily at GC sites in a variety of mutagenicity assays, supporting the

view that MX-induced damage at guanine may be the critical lesion that is processed into MX-induced mutations.

Knasmüller et al. (1996) conducted a study comparing the mutagenic potency and specificity of MX with the related compounds, CMCF (3-chloro-4-(chloromethyl)-5-hydroxy-2(5H)-furanone), MCA (3,4-dichloro-5-hydroxy-2(5H)-furanone), and chloromalonaldehyde, a putative breakdown product of MCA. Because MX and the other tested compounds showed greater mutagenic potency in strains with *mucAB*-encoded error-prone repair (strains carrying the pKM101 plasmid) than in corresponding strains without the *mucAB* genes, the authors concluded that these chlorinated furanones are SOS repair-dependent mutagens. In this study, CMCF produced primarily GC → TA transversions, but to a lesser degree than MX, whereas MCA and CMA produced primarily GC → AT transitions. Hyttinen et al. (1995) observed similar results with MX, CMCF, and MCA tested in *Salmonella* TA100, supporting the view that changing substituents on the furanone ring changes not only mutagenic potency but also mutagenic specificity.

To probe the nucleoside specificity of MX and its analogues in relation to mutagenicity, a recent study examined MX, MCF, and 7 structurally related compounds in *Salmonella* strains TA98, TA100, and TA104 (Franzén et al., 1998). MCF was twice as mutagenic in TA104, which presents both GC and AT targets for mutagenesis, as it was in TA100, which presents primarily GC targets (Franzén et al., 1998). In contrast, MX was found to be almost 30% less mutagenic in TA104 compared with TA100. Based on these results, Franzén et al. (1998) inferred that MCF induced mutations at AT sites more frequently than did MX.

Molecular analysis of MCF-induced mutations, which would support or

refute the assumptions of greater targeting of adenosine by MCF compared with MX, have not yet been reported. Therefore, in the present study we have determined the mutation spectrum of MCF in relation to MX to determine if the spectra of these two compounds are (a) consistent with the reported nucleoside adducts formed by these mutagens and (b) explain the dramatic difference in mutagenic potency of the two compounds.

Materials and Methods

Chemicals

Magnesium sulfate (6066), citric acid monohydrate (0627), anhydrous dibasic potassium phosphate (7092), and sodium ammonium phosphate (8124) were obtained from Mallinkrodt (Paris, Kentucky). Materials purchased from Sigma included glucose-6-phosphate monosodium salt (G-6-P, G-7879), nuclease-free bovine serum albumin (BSA, A 4503), ethidium bromide (E 8751), nuclease-free ficoll (F 4375), polyvinylpyrrolidone (P 5288), hydrochloric acid (251-02), d-Biotin (B4512), L-Histidine (H 8125), magnesium chloride (M 9272), methylglyoxal (M 0252), and sodium azide (S 8032).

Tris ultrapure electrophoresis grade (22643) and urea ultrapure electrophoresis grade (23040) were bought from United States Biochemicals (USB, Cleveland, Ohio). Calf thymus DNA (2168) was purchased from Calbiochem (San Diego, Ca). Difco Brain Heart Infusion (BHI) agar (0418), Difco agar (0140-01), dextrose (B155), and filter papers (F2413, F2605-1, and F2600-1) were purchased from Baxter (McGaw Park, Il). Oxoid nutrient broth No.2 (CM67) was obtained from Oxoid Ltd. (Basingstoke, Hampshire, England).

Materials obtained from Biorad (Richmond, CA) included ammonium persulfate (161-0700), xylene cyanol FF (161-043), bromophenol blue (161-0404), and N,N,N'-N'-tetra methylethylenediamine (TMED, 161-0801).

Formamide (0606) was purchased from Ameresco (Solon, OH) and dimethyl sulfoxide (DMSO) Burdick and Jackson by high purity solvent was purchased from American Scientific Products (McGaw Park, IL). Materials obtained from Fisher Scientific (Fairlawn, NJ) included disodium ethylenediamine tetra acetate (EDTA, S311-500), potassium chloride (P217), sodium chloride (S271-500), and boric acid (A74-500).

Long Ranger Singel™ Packs were obtained from FMC BioProducts (Rockland, Me). Kodak X-OMAT AR X-ray film was supplied by Lab Source Inc. (Chicago, IL). Probes and primers were purchased from Operon Technologies (Alameda, Ca), and γ -³²P dATP was supplied by Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Nacs Prepac™ cartridges (1526NP) were purchased from Bethesda Research Laboratories (Gaithersburg, Md).

MX and MCF (Figure 1) were synthesized according to methods described previously (Franzen and Kronberg, 1995) and were provided as a gift from Dr. Robert Franzen (Department of Pharmacy, University of Helsinki, Helsinki, Finland) and Dr. Leif Kronberg (Department of Organic Chemistry, Åbo Akademi University, Turku/Åbo, Finland).

Solutions

Vogel-Bonner medium E (VBME) 50x salt solution was prepared as follows: 10 g of magnesium sulfate, 100 g of citric acid monohydrate, 500 g of potassium phosphate dibasic anhydrous, and 175 g of sodium ammonium phosphate were dissolved in 670 ml of deionized, distilled water at 45°C. The solution was then filtered through Whatman #1 filter paper and autoclaved for 20 minutes at 121°C. Dextrose solution for use in VBME and biotin plates contained 30 g of dextrose dissolved in 100 ml of

deionized, distilled water. Histidine/biotin solution used in VBME plates was prepared as follows: 97.6 g of d-biotin were dissolved in 1000 ml of deionized water preheated to 45°C. After the solution was cooled, 52.5 mg of sterile L-histidine-HCl were added.

Denhardt's solution (50x) was prepared by dissolving 5 g of ficoll, 5 g of polyvinylpyrrolone, and 5 g of bovine albumen in 500 ml of deionized, distilled water, and the solution was filter-sterilized. Sodium chloride-sodium citrate solution (20X SSC) containing 701.2 g of sodium chloride and 352.8 g sodium citrate dissolved in 3000 ml of deionized water, was pH adjusted to 7.0 with 37% HCl, and was autoclaved.

Media

Vogel-Bonner Medium E (VBME, Maron and Ames, 1983) was prepared by adding 45 g of Bacto-agar (Difco) to 2655 ml of deionized water. The solution was heated to dissolve the agar, autoclaved for 20 minutes at 121°C, and 200 ml of sterile dextrose solution were added. After the medium was cooled to 48°C, 60 ml of sterile 50x VBME salt solution and 30 ml of sterile histidine/biotin solution were added aseptically. The agar was dispensed into sterile petri plates in quantities of approximately 30 ml by means of an automatic plate pourer.

Biotin medium was prepared by dissolving 45 g of Bacto-agar (Difco) in 2655 ml of preheated deionized water. The agar solution was cooled to 48°C, and 200 ml of dextrose solution, 60 ml of sterile 50X VBME solution, and 30 ml of sterile biotin solution were added aseptically.

Bacterial Strains

Salmonella typhimurium strains TA100 (*hisG46*, *rfa*, *uvrB*, *pKM101*) and TA104 (*hisG428*, *rfa*, *uvrB*, *pKM101*) were provided by Dr. Bruce Ames, University of California, Berkeley. The *hisG46* allele in TA100 contains a mutation in the *hisG* gene, which codes for ATP phosphoribosyltransferase, the first enzyme in the histidine biosynthetic pathway (Ames, 1971). The *hisG46* mutation was isolated from a spontaneously arising AT $\rightarrow$ GC transition in *S. typhimurium* LT2 at the *hisG* codon 69 (position 434), which changes a leucine codon (CTC) to a proline codon (CCC). A variety of base substitutions at the first and second positions of the *hisG46* CCC codon can revert cells to *His*⁺ prototrophy (Hartman et al., 1971). Mutations at extragenic suppressor loci, e.g., specific mutations in the anticodon regions of tRNA molecules, can also revert *hisG46* mutants (Levin and Ames, 1986). The *hisG428* allele in TA104 is an ochre mutation, TAA, in the *hisG* gene. The AT base pairs in the mutational target permit the detection of a variety of oxidative mutagens, including x-rays, bleomycin, hydrogen peroxide, formaldehyde, and glyoxal. The *hisG428* allele is reverted by a variety of mutations within the TAA target and by several known suppressor mutations.

Other mutations in TA100 and TA104 include deep rough (*rfa*), which results in a deletion in the lipopolysaccharide barrier that coats the outer membrane of the bacteria, thereby increasing permeability to large molecules that normally do not penetrate the cell wall. The Δ *uvrB* mutation is a deletion of a gene in the DNA excision repair system that encodes one subunit of an endonuclease that recognizes DNA damage, resulting in distortions of the DNA helix. This deletion extends through

the *bio* gene as well; consequently, bacteria with the *uvrB* mutation require biotin for growth. The R-factor plasmid pKM101 contains *mucA* and *mucB* genes that enhance an error-prone repair system that is normally present in *S. typhimurium* (Maron and Ames, 1983).

Salmonella Plate-Incorporation Mutagenicity Assay

The plate-incorporation mutagenicity assay was performed as described by Maron and Ames (1983). Overnight cultures of TA100 and TA104 were prepared. Sodium azide and methyl glyoxal were used as the positive controls for TA100 and TA104, respectively. DMSO was used as the negative control for each strain. MCF was tested in both TA100 and TA104 at doses ranging from 2 μ g/plate to 32 μ g/plate, and MX was tested in TA104 at a dose range of 0.05 μ g/plate to 0.5 μ g/plate. Mutagenicity of MCF and MX was evaluated by adding 100 μ l of bacterial cells and the appropriate dose of test chemical to molten top agar. The mixture was vortexed, added to VBME agar plates, and allowed to solidify. Plates were inverted and incubated for 72 hours at 37°C. Compounds were tested in triplicate for each dose, and the number of revertants was expressed as the mean of three plates per dose. Revertant colonies were counted with an automatic plate counter (BioTran III).

Colony Probe Hybridization

Colony probe hybridization was performed to determine the mutational specificity of MX and MCF. The colony probe hybridization technique, first developed for the *hisG46* allele by Miller and Barnes

(1986) and modified by Cebula and Koch (1990), permits the rapid screening of mutants for six common mutations in TA100. Adaptation of this method by Koch et al. (1996) permits detection of ten common mutations in TA104. Hybridization is the pairing or annealing of two complementary strands of DNA to form a hybrid of two single strands of DNA. In the colony hybridization technique, DNA from *Salmonella* tester strains is denatured using an alkaline solution and heat-fixed to filter paper yielding single-stranded DNA. Probes are single-stranded 15-20 base oligonucleotide sequences, labeled at the 5' end with γ -³²dATP, containing common base substitutions found in TA100 and TA104 revertants. After allowing the probes to hybridize to single-stranded DNA fixed to the filter paper under stringent conditions, excess radioactive probe is washed from the filter. Filters are dried and exposed to x-ray film to generate autoradiograms. Revertant colonies containing one of the variety of common base substitutions will hybridize to the corresponding probe and appear as black spots on the autoradiogram.

The five intragenic base substitutions that revert the *hisG46* allele in TA100 to *His*⁺ prototrophy are ACC, TCC (GC → TA and GC → AT at position 1 of the CCC missense codon) CAC, CTC (GC → TA and GC → AT at position 2), and GCC (GC → CG at position 1). The *hisG428* allele is reverted by the following base substitutions: GAA, TCA, and TAC (AT → CG at positions 1, 2, and 3, respectively), AAA, TTA, and TAT (AT → TA at positions 1, 2, and 3, respectively), CAA (AT → GC at position 1), and by three suppressor mutations, *SupB* (GC → AT), *SupCM* (GC → TA), and *SupG* (AT → TA).

Colonies were picked for molecular analysis at doses yielding at least twice the number of induced revertants compared with revertants of

spontaneous origin. Single colonies were streaked onto minimal medium supplemented with biotin to isolate and purify colonies from the background lawn (Maron and Ames, 1983). Filters containing single-stranded DNA from individual colonies were prepared according to methods described by Cebula and Koch (1990). Individual colonies were inoculated into microtiter plates containing 200 μ l of nutrient broth in each well, incubated for 24 hours at 37°C, and replicated onto brain-heart infusion (BHI) agar plates. After overnight incubation, all colonies from a single BHI plate were transferred to a Whatman 541 paper filter and placed in 5 ml of M1 solution (0.5-M NaOH, 1.5-M NaCl) for 5 minutes. The filters were placed in glass petri dishes, heated in a microwave oven (set on "Defrost") for 30 seconds, and transferred to petri plates containing 10 ml of M2 solution (1-M Tris, 2M-NaCl, pH 7.0) for 5 minutes. After air drying, the filters were ready for hybridization with radioactive probes.

Probes were prepared with 5 μ l of each oligonucleotide (diluted to a concentration of 4 pmoles/ μ l), 5 μ l of γ -³²ATP, and 1 μ l of T4 polynucleotide kinase. The mixture was heated for 10 minutes at 37°C and for 2 minutes at 90°C. The radiolabeled probes were then purified using NACS Prepac™ cartridges. For probe hybridization, filters were placed in petri plates containing 10 ml of hybridization solution consisting of SSC (0.15-M NaCl, 0.015-M sodium citrate), Denhardt's solution (Maniatis et al., 1982), and calf thymus DNA. Calf thymus DNA was added to prevent nonspecific binding of the radiolabeled probes to the filter paper. After adding ~5 μ l of radiolabeled probe to each petri plate, filters were incubated for 2 hours on a rotating platform set to low shaking speed and then washed (at various temperature settings and timings for each probe

based on stringency conditions established for each oligonucleotide sequence) with pre-warmed SSC solution to remove unhybridized probe. The filters were dried and placed on x-ray film overnight at -80°C.

Polymerase Chain Reaction (PCR)

Colonies that failed to hybridize were subjected to polymerase chain reaction (PCR) amplification and DNA sequencing according to methods described by Bell et al. (1991). For PCR, oligonucleotide primers (15-30 bases) were designed to anneal to regions in the template DNA that bracket the DNA sequence to be amplified. Repeated cycles of heating at different temperatures allow for denaturation of double-stranded DNA, annealing of primers, and extension of the primers by the Taq polymerase. Primers were added (in excess of the DNA to be amplified) to a reaction mixture consisting of buffer containing MgCl₂ (Promega, M-1883) deoxyribonucleoside triphosphates (dNTP's, Perkin-Elmer, N808-0007), and Taq enzyme (ProMega, M-188A).

DNA from revertant colonies was isolated as follows. Colonies were isolated and purified from the plate-incorporation assay by restreaking onto biotin plates and incubated for 24 hours. Individual colonies were transferred to pre-labeled 0.5-ml tubes containing 200 µl of sterile deionized, distilled water, boiled for 10 minutes at 100°C, and centrifuged (10,000g) for 10 minutes. Approximately 5 µl of the supernatant containing *Salmonella* genomic DNA were added to the 45 µl of the PCR reaction mixture. Symmetric PCR of *hisG46* revertants was performed using a Perkin-Elmer Cetus 9600 thermal cycler in which the PCR reaction mixture was heated initially for 5 minutes at 85°C followed by 25

cycles of 15 seconds at 96°C, 15 seconds at 50°C, and 40 seconds at 72°C. The sequence of the two primers (G1 and G2) were G1 (5'-GAT TCA CGA GAA TTG CTG GC-3') and G2 (5'-GGA TAT GAG GTA GCG ATA CG-3'). For PCR amplification of *hisG428* revertants, the primers used were TA104-1 (5'-GCG ACG CTT GAA GCT AAC G-3') and TA104-2 (5'-CTT GGC GCG TGC ATC ATG A-3'). For *hisG428* revertants, the PCR reaction mixture was heated initially for 5 minutes at 85°C followed by 35 cycles of 15 seconds at 94°C, 20 seconds at 55°C, and 40 seconds at 72°C.

The presence of the 350-base pair product was monitored by electrophoresis of 5 µl of the reaction mixture on an agarose gel composed of 1% agarose (Gibco, Grand Island, NY), Tris-borate-EDTA buffer, and ethidium bromide. Excess primers and dNTPs were removed by ultrafiltration using Centricon-100 microconcentrators (Amicon, Danvers, Ma) in preparation for dideoxy sequencing.

Dideoxy Sequencing

The Sanger method for sequencing single-stranded DNA (Sanger, 1977) relies on the inhibitory activity of dideoxynucleotide triphosphates (ddNTPs) incorporated into the growing oligonucleotide chain on the DNA polymerase. Because ddNTPs contain no 3' hydroxyl group, no additional deoxynucleotides can be incorporated, and the growing oligonucleotide chain is terminated. In the sequencing reaction, a mixture of dNTPs and ddNTPs, in a ratio that guarantees incorporation of a ddNTP into each position occupied by the corresponding dNTP, is incubated with the DNA polymerase. The sequencing reaction yields fragments of varying lengths that are separated by electrophoresis on an acrylamide gel. Sequencing

was performed as described by the manufacturer using Big Dye termination mix (PE Applied Biosystems, Foster City, Ca). A 10- μ l reaction mixture consisting of 2.5 μ l of purified PCR product and 7.5 μ l of sequencing mixture (4 μ l of Big Dye termination mix, 0.48 μ l of TA104-1 primer at 10 pm/ μ l, and 3.02 μ l of sterile deionized water) was heated for 25 cycles of 10 seconds at 96°, 5 seconds at 50°C, and 4 minutes at 60°C. Excess primer and termination mix were removed using CentriSep columns (Princeton Separations, Adelphia, NJ). The eluate was evaporated to 20 μ l, 5 μ l of loading/formamide were added, and 1.5 μ l of sample were loaded and sequenced on an ABI 377 Automated Sequencer.

Statistical Analysis

The base-substitution spectra from TA100 were compared to a laboratory historical control from DeMarini et al. (1995) by Π^2 analysis using the Stat-Sak program (Gerald E. Dallal, Malden, MA). For this analysis, the five categories of single base-substitutions were collapsed into two categories, GC $\rightarrow$ TA transversions and GC $\rightarrow$ AT transitions. Π^2 analysis was also used to compare the spectra from TA104 to a published spontaneous spectrum (Koch et al., 1996) that is not significantly different from our own unpublished laboratory control ($P = 0.3$).

RESULTS

Dose-response curves for revertants induced by MCF in TA98, TA100, and TA104 and by MX in TA104 are shown in Figure 6. For cells treated with MCF, revertants were picked for molecular analysis at a dose of 32 μ g/plate in both TA100 and TA104, yielding a fold increase of 10.9 and 3.5, respectively (Table I). The fold increase was calculated by dividing the number of revertants induced by a mutagen by the number of spontaneous revertants. Based on these fold increases, 91% of the MCF/TA100 revertants and 71% of the MCF/TA104 revertants were induced as opposed to being spontaneous in origin. MX/TA104 revertants were picked at a dose of 0.5 μ g/plate with a fold increase of 2.4. Thus, 58% of the revertants were induced by MX as opposed to being spontaneous in origin.

As shown in Figure 7, both MCF and MX produced primarily GC $\rightarrow$ TA transversions (75% and 86%, respectively) in TA100. Most of the remaining mutations were GC $\rightarrow$ AT transitions (20% and 11%, respectively). The spectrum of MCF in TA100 was significantly different from the background ($p = 0.03$) as was that of MX ($p < 0.001$). Table II gives the frequency of classes of mutations that can be recovered in TA100. CCC is the target sequence of the *hisG46* allele, and revertants containing this sequence represent extragenic suppressor TA $\rightarrow$ GC

Dose response of MX and MCF in TA98, TA100, and TA104

Figure 6

TABLE I. Conditions Under Which Mutants Were Selected

Chemical	Strain	Dose	Rev/plate	Fold	No. rev
		(μ g/plate)	(treated/spont.)	increase	analyzed
MCF	TA100	32.0	1020/94	10.9	102
	TA104	32.0	856/245	3.5	108
MX	TA100 ^a	0.4	2030/86	23.0	200
	TA104	0.5	702/297	2.4	119

^a from DeMarini et al. (1995)

Percentage of the Two Primary Classes of Base-Substitutions
Induced by MX and MCF in TA100 of Salmonella

Figure 7

TABLE II. Frequency of Background and Induced Revertants of the *hisG46* Allele

Agent	Frequency (%) of mutational event						Probe
	TCC	ACC	GCC	CTC	CAC	CCC ^a	negative ^b
BKG ^c	17.5	16.5	3.5	17.0	43.0	0.5	2.0
MCF	7.8	15.7	0.0	11.8	58.8	0.0	5.9
MX	4.0	22.0	0.0	7.0	63.5	0.0	3.5

^aCCC is the target sequence of the *hisG46* allele, and revertants containing this sequence represent extragenic suppressor TA → GC transversions of either of two tRNA^{thr} genes (Kupchella et al., 1994).

^bProbe-negative revertants were those that did not hybridize to any of the six probes and, presumptively, contained multiple mutations (Koch et al., 1994); these mutants were not sequenced.

^cBackground (spontaneous) data are laboratory historical control data from (DeMarini et al., 1995). Background vs. MCF, $p = 0.03$.

TABLE III. Frequency (%) of Background and Induced Revertants at the *hisG428* Allele

Mutational event	BKG ^a	MX	MCF
Suppressors			
<i>SupB</i> , GC → AT	5.0	0.8	5.6
<i>SupG</i> , AT → TA	6.0	0.8	1.9
<i>SupC/M</i> , GC → TA	53.0	84.9	57.4
Other	12.0	-- ^b	-- ^b
Intragenic			
CAA, AT → GC	1.5	0.0	0.9
AAA, AT → TA	9.0	5.9	3.7
TTA, AT → TA	7.0	5.0	14.8
TAT, AT → TA	2.5	0.0	
GAA, AT → CG	0.5	0.8	0.9

TCA, AT → CG	0.5	0.0	1.9
TAC, AT → CG	1.0	0.0	0.0
Other	2.0	— ^b	— ^b

^aBackground (spontaneous) spectrum from (Koch et al., 1996); it is not significantly different ($p = 0.23$) from our unpublished laboratory historical control spectrum.

^bThere were 2 MX revertants (1.7%) and 2 MCF revertants (1.9%) that did not hybridize with any probes. They were not available for sequence analysis because they had been discarded and, thus, could not be categorized genetically.

transversions of either of two tRNA^{trp} genes (Kupchella et al., 1994). The background spectrum from laboratory historical control data (DeMarini et al., 1995) consisted of 60% GC → TA transversions and 35% GC → AT transitions. The mutation spectra of MCF and MX in TA100 were not significantly different from each other ($p = 0.07$).

The distribution of revertants recovered in TA104 after treatment with either MX or MCF is shown in Table III. The vast majority of mutations induced by MX were at suppressor (tRNA) genes, whereas MCF-induced mutations were distributed more equally between intragenic and suppressor mutations (Table IV and Figure 8). Classification of induced and background mutations between suppressor and intragenic mutations resulted in spectra that were significantly different ($p < 0.001$). Mutations were significantly different between MX and MCF, based on categories of suppressor mutations or intragenic mutations ($p = 0.04$ and 0.02 , respectively).

The distribution of revertants among the five classes of mutations that can be recovered in TA104 is shown in Table V. The mutation spectra produced by MCF and MX in TA104 were significantly different from each other ($p < 0.001$). The observed mutation spectra were subtracted from the expected background spectra to produce the induced spectra shown in Table VI. The expected number of background mutations was calculated as follows: first, the number of mutants analyzed was divided by the fold increase (i.e., the increase over background) to give the estimated number of revertants of spontaneous origin, namely

Table IV. Estimated Frequency (%) of Intragenic and Suppressor Mutations Induced at the *hisG428* Allele

Agent	Class	Bkg. (%) ^a	Expected	Observed	Induced (%)
MX	Intragenic	23	11.4	14	2.6 (3.9)
	Suppressor	77	38.2	103	64.8 (96.1)
MCP	Intragenic	23	7.1	36	28.9 (38.5)
	Suppressor	77	23.8	70	46.2 (61.5)

^aBackground data from (Koch et al., 1996).

Estimated Frequency (%) of Induced Intragenic and Suppressor Mutations in TA104 After the Background Has Been Subtracted

Figure 8

TABLE V. Distribution (%) of Revertants Among the Five Classes of Base Substitutions at the *hisG428* Allele

Mutation	Background ^a	MX	MCF
GC → AT	5.0	0.8	5.6
AT → GC	1.5	0.0	0.9
GC → TA	53.0	84.9	57.4
AT → CG	2.0	0.8	2.8
AT → TA	24.5	11.7	31.5

^aBackground data from (Koch et al., 1996). Background vs. MX, $p < 0.003$; background vs. MCF, $p = 0.93$; MX vs. MCF, $p < 0.001$.

TABLE VI. Estimated Number of Induced Mutations at the *hisG428* Allele

Agent	Mutation class	BKG (%) ^a	Expected ^b	Observed ^c	Induced (%)
MX	GC → AT	5.0	2.5	1	--
	AT → GC	1.5	0.7	0	--
	GC → TA	53.0	26.3	101	74.7 (98)
	AT → CG	2.0	1.0	1	--
	AT → TA	24.5	12.1	14	1.9 (2)
	Other	14.0	6.9	2	--
MCF	GC → AT	5.0	1.5	6	4.5 (6)
	AT → GC	1.5	0.5	1	0.5 (1)
	GC → TA	53.0	16.4	62	45.6 (57)
	AT → CG	2.0	0.6	3	2.4 (3)
	AT → TA	24.5	7.6	34	26.4 (33)
	Other	14.0	4.3	2	--

^aFrom (Koch et al., 1996).^bThe expected number of background mutations in each spectrum was calculated

by first dividing the total number of mutants analyzed by the increase over background to obtain the estimated number of background mutations in the spectra. This number was then distributed among the mutant classes according to the frequency of the occurrence of the mutant classes in the background mutation spectra.

Data can be calculated from the percentage distribution in Table IV and the actual number of mutants analyzed and the fold increase in Table I.

30.9 background mutations out of 108 total revertants for MCF and 49.6 background mutations out of 119 for MX. These numbers were then distributed among the six classes of mutations according to the frequency (%) at which they occur. The expected number of revertants was then subtracted from the observed number to give the induced number of revertants in each of the classes of mutations. The results show that in TA104 MX induced GC → TA transversions almost completely (98%), with the remaining revertants (2%) being AT → TA transversions. For MCF, 57% of the induced mutations were GC → TA transversions, with AT → TA transversions accounting for 33% of the remaining induced mutations. Consequently, nearly all of the revertants induced by MX (98%) were at GC sites, whereas only 63% of the mutations induced by MCF were at GC sites (Figure 9).

Percentages of Mutations at AT sites vs. GC Sites
in TA104 After the Background has Been Subtracted

Figure 9

Discussion

Structural Effects of MX and MCF on Mutagenic Potency and Specificity

The results of this study demonstrate that the replacement of a 4-methyl group in MCF (in the closed form) with a 4-dichloromethyl group in MX not only dramatically increases mutagenic potency but also shifts the mutagenic specificity from almost equal targeting of GC and AT sites by MCF to an almost exclusive targeting of GC sites by MX. Nonetheless, MX and MCF share a number of reactivity and structural similarities. Both compounds can exist in a closed ring form at low pH or an open oxobutenoic form at higher, physiological pH (Figure 1). Evidence from UV spectra show a shift from 223 nm at pH <6.0 to 254 nm at pH >6.0, indicating that these compounds adopt primarily the open form at pH >6.0 (Munter et al., 1996, 1999). In the open form, both compounds are halogenated ethylenes with terminal carboxylic acid and aldehyde groups, and it is the open form containing the free aldehyde group that is considered the active form (Munter et al., 1996, 1999). For both MX and MCF, mechanistic evidence from ¹³C-labeling experiments and the results of nucleoside experiments (shown in Figure 2) point to the initial attack of the exocyclic amino group of adenine or guanine on the same labeled carbon atom, namely the 3-methyl carbon of MCF (in the open form) or the 3-dichloromethyl carbon of MX (also in the open form).

The few structural and reactivity differences between MX and MCF

must, therefore, explain the dramatic differences in mutagenic potency and specificity between the two compounds. MX is significantly more electrophilic than MCF according to the calculated frontier orbital energy (ELUMO) values, and this measure of electrophilicity was found to correlate strongly with the mutagenic potency of a series of compounds related structurally to MX in TA100 (Tuppurainen and Lötjönen, 1993). From the hypothesized adduct data shown in Figure 2, the carbon centers that differ most in MCF and MX, namely the 4-methyl and 4-dichloromethyl carbons respectively, are the sites on these compounds that react with guanosine and adenine. This effectively accounts for the reactivity differences between these otherwise structurally similar compounds.

Both MX and MCF have been found to react (as direct-alkylating agents) exclusively with the exocyclic nitrogens of adenine and guanine (Figure 2). This is a feature typical of compounds having extended conjugation systems, such as the epoxide metabolites of polycyclic aromatic hydrocarbons (PAHs), which also act as direct-acting alkylating agents. For PAHs, reactivity is thought to be related to the ability of PAHs to delocalize charge and stabilize a carbocationic charge at the site of nucleophilic attack (Moschel et al., 1979; Dipple et al., 1982). However, MX and MX-related compounds bear little structural resemblance to PAHs, and a carbocationic reactive intermediate is unlikely for MX-related compounds. Both MX and MCF have electronegative groups centered around an ethylene bond, which may increase susceptibility for nucleophilic attack by bases.

Based on this study, MX targets GC sites almost exclusively in TA104, whereas only 63% of mutations induced by MCF were at GC sites.

Most of the remaining mutations induced by MCF (33%) were at AT sites. A previous study of MX conducted by Knassmüller et al. (1996) in a strain related to TA104 (but missing the pKM101 plasmid) showed results similar to ours, namely a predominance of GC → TA transversions with a small percentage of AT → TA transversions. Similar studies of MX analogues in *Salmonella* TA100 (Hyttinen et al., 1995) have demonstrated that another disinfection by-product similar in structure to MX, 3-chloro-4-(chloromethyl)-5-hydroxy-2(5H)-furanone (CMCF), induces primarily GC → TA transversions (Figure 10). The results of the study by Hyttinen et al. (1995) demonstrate that alterations of the structure at the 4-carbon of MX analogues change mutational specificity. With a 4-dichloro-methyl group in MX, mutations were primarily GC → TA transversions. CMCF, which has a 4-chloromethyl group, again induced primarily GC → TA transversions (76%), although the number of GC → AT transitions is increased to 23%. In the mutation spectra of MCA, in which the 4-dichloromethyl group of MX is replaced by a 4-chloro group, GC → AT transitions predominate (66%) with the remaining mutations (33%) being GC → TA transversions. Although MCA has a different carbon backbone than MX, CMCF, or MCF, clearly when substituents at the 4-carbon position of the furanone are altered the mutation spectra in TA100 are altered as well. Although the mutation spectra of CMCF in TA104 has not been reported, based on structural considerations and mutagenicity data, we would predict CMCF to induce mutations predominately at GC sites, similar to MX. Recent findings that CMCF forms two adducts with guanine support this prediction (Kronberg, in preparation).

Percentage of the Two Primary Classes of Mutations
in TA100 Induced by MX, CMCF, and MCA

	MX	CMCF	MCA
GC → TA	MX 87 ^a	CMCF 75	MCA 32
GC → AT	11	23	66

^apercentage of reversion events at the *hisG46* allele in TA100
(Hytinen et al., 1995)

Figure 10

Possible Adduct Formation

From the adduct studies shown in Figure 2, MX and MCF produced guanosine adducts only when reacted with individual nucleosides; no guanosine adducts were detected when MX or MCF was reacted with calf thymus DNA. In addition, when both MX and MCF were reacted with guanine, only MX produced detectable adducts. The almost exclusive targeting of GC sites by MX in TA104 supports a possible mutagenic role for the guanosine adduct shown in Figure 2 (Munter et al., 1999). The lack of detectable guanosine adducts may be explained by the formation of apurinic sites at guanines. Munter et al. (1999) proposed that apurinic sites may occur as a consequence of adduct formation on guanine followed by cleavage of the glycosidic bond and release of the adducted guanine. Recent evidence that MX induces apurinic sites when reacted with DNA has been reported by Hyttinen and Jansson (1995). The formation of a highly unstable adduct that results in apurinic sites may explain the predominance of MX- and MCF-induced GC → TA transversions observed in the present study and in all the genetic systems in which they have been studied so far. Adenine is incorporated most frequently opposite noncoding lesions (e.g., apurinic sites), and such sites are known to be processed predominantly into GC → TA transversions (Kunkel, 1984). Finally, Tupparainen (1997) has proposed a mechanism (not involving adducts) consistent with targeting of GC sites by MX followed by depurination that operates by a one-electron reduction at GC sites in DNA.

The MX-adenosine adducts identified in reactions with DNA, MX-A2, and MX-A3 in Figure 2 (LeCurieux et al., 1997; Munter et al., 1998) could

account for the small percentage of MX-induced mutations at AT sites in TA104. LeCurieux et al. (1997) reacted MCF with calf thymus DNA, and only MCF-A2 was detected in the hydrolyzed DNA; MCF-A1 was not detected. The MCF-A2 adduct could account for the finding that 33% of the mutations induced by MCF in TA104 were AT → TA transversions.

Despite the extensive adduct and mutation spectra studies on MX and MX-related compounds, a variety of questions remain to be answered. What are the rates of repair and mutagenic efficiencies of the various adducts and apurinic sites? Are the final adduct structures formed equally, and are the relative efficiencies determined by the rate of adduct formation? Are the rates and efficiencies of adducts likewise determined by the response of the DNA polymerase to the different adducts? The nature of the interaction between MX and (possibly MCF) and DNA that leads to apurinic sites has yet to be determined. Finally, given the close structural similarity between MX-A3 and MCF-A2, why doesn't MX-A3 produce similar levels of AT → TA transversions as MCF-A2 presumably does?

The Roles of MX and MCF in the Overall Mutagenicity of Drinking Water Extracts

Although chlorinated hydroxyfuranones and related compounds are among the most mutagenic compounds identified drinking water extracts (Kronberg et al., 1991; Kronberg and Franzen, 1993), collectively they account for less than 50% of the total mutagenicity of a drinking water extract (Franzen and Kronberg, 1994). MCF, while present in concentrations similar to MX, is substantially less potent than MX and accounts for at most 1% of the mutagenic activity in drinking water

extracts (Smeds et al., 1997). The contribution of MX to the overall mutagenicity of organic extracts of drinking water has been estimated to be only about 20%, based on an estimated MX concentration of 15 ng/L for samples of drinking water that were disinfected with chlorine (DeMarini et al., 1995). Therefore, other compounds that can account for the remaining mutagenicity may include chlorinated compounds already present in the raw water before disinfection, including discharge from waste-water treatment plants, effluent discharge from industry, agricultural runoff containing chlorinated pesticides, and compounds settling from air containing emissions from combustion of chlorinated organics (Haglund et al., 1987; Oehme et al., 1987; Coleman et al., 1992; Houk et al., 1992). In the presence of high bromide-ion concentrations, chlorination of organic compounds produces brominated and mixed halogen analogues of MX. The mutagenicity of some of these brominated counterparts of MX are comparable to MX and CMCF (LaLonde et al., 1997). Perhaps a combination of chlorinated and brominated compounds related to MX, along with additional unidentified compounds, contribute to the remaining mutagenicity of organic extracts of drinking water.

In a recent study conducted in our lab, mutation spectra in *Salmonella* TA98, TA100, and TA104 were determined for four chlorinated butenoic acids related structurally to MX (Landi et al., submitted). Although these butenoic acids have not yet been detected in drinking water extracts, the spectra of these BA compounds in TA98 and TA100 were found to be similar to that of an organic extract of drinking water, but significantly different from that of MX, indicating that compounds related structurally to these chlorinated butenoic acids may account for much of

Structures and Chemical Names of
Butenoic Acids Related to MX

BA-1

3-chloromethyl-4,4-
dichlorobutenoic acid

BA-2

3-dichloromethyl-4,4-
dichlorobutenoic acid

BA-3

2,4,4-trichloro-3-
chloromethylbutenoic acid

BA-4

2,4,4-trichloro-3-
dichloromethylbutenoic acid

Figure 11

the mutagenic specificity of organic extracts of drinking water. Although the spectrum for the chlorinated drinking water extract was not determined in TA104, comparisons of these spectra with those of MX and MCF showed a significant difference in site specificity between MX and the other compounds. Two of the butenoic acids containing chloromethyl groups, BA-1 and BA-3 (Figure 11), induced primarily AT $\rightarrow$ TA transversions (61-63%), indicating that these compounds may target AT sites as a consequence of having a chloromethyl group at the 3-carbon, inducing a relatively high percentage of AT $\rightarrow$ TA transversions. In contrast, BA-2 and BA-4, which have a dichloromethyl group at the 3-carbon, induced a higher percentage of GC $\rightarrow$ TA transversions (45-48%), with AT $\rightarrow$ TA transversions accounting for the 38-47%. The dichloromethyl group found in BA-2 and BA-4 may have a role in the greater targeting of GC sites by these compounds.

Overall Effects of MX and MCF in Vivo

Many of the studies aimed at detecting genotoxic activity of drinking water extracts have been conducted in vitro, primarily with bacterial test systems, but also with mammalian cell cultures (Meier, 1988). In vivo studies, in which drinking water concentrates were administered to mice at doses approximating 1, 3, and 10 years of human consumption, yielded negative results when bone marrow cells were analyzed for the induction of micronuclei (Wilcox and Williamson, 1986; Meier et al., 1987). In both studies, drinking water concentrates at the same doses as the in vivo study were found to be genotoxic in bacterial and mammalian cells in vitro. Likewise, chlorinated humic acid, when administered to mice by oral gavage, failed to induce micronuclei or

sperm-head abnormalities, but when tested in vitro induced mutations in *Salmonella* and sister-chromatid exchanges in Chinese hamster ovary cells (Meier and Bull, 1985).

The discrepancy in results between in vivo and in vitro assays can be attributed to metabolic detoxification by the liver in the rodent studies. The mutagenicity of drinking water extracts is substantially reduced in vitro by rodent liver S-9 homogenate (Meier et al., 1986). Likewise, numerous studies in vitro have demonstrated that the mutagenic effects of MX are dramatically reduced when tested in combination with S-9 homogenate (Ishiguro et al., 1988; Meier et al., 1987; Kronberg et al., 1991). In general, evidence that mammalian cells may effectively detoxify MX is derived from comparisons of the relatively weak genotoxic effects of MX in vivo compared to the potent mutagenicity of MX in *Salmonella* assays. Inactivation of MX and related compounds by S-9 homogenate may also be due to nonspecific protein binding. Brunberg et al. (1991) have attributed the lack of MX-induced DNA damage in stomach epithelium to the interaction of MX with macromolecules such as stomach mucin. MX has been shown to bind avidly to serum albumen (Haataja et al., 1991); therefore, much of the MX in chlorinated drinking water may bind to cellular proteins before being able to interact with DNA.

Sulfhydryl compounds, including glutathione, L-cysteine, cysteamine, and N-acetylcysteine (NAC), have been shown to substantially reduce mutagenicity induced by MX and MX-related compounds in bacteria (LaLonde and Xie, 1993; Watanabe et al., 1994). In the study by Watanabe et al. (1994), sulfhydryl compounds, including glutathione, L-cysteine, and cysteamine, were effective in reducing the number of MX-induced

revertants, but antioxidants such as L-ascorbic acid and α -tocopherol had no antimutagenic effect against MX. Displacement of chlorine or dichloromethyl groups by sulfur in thiols is thought to be the mechanism by which the mutagenicity of MX and related compounds is reduced (LaLonde and Shie, 1992). In one example, mutagenicity of MCA in *Salmonella* TA100 was reduced by NAC. The major product resulting from the displacement of the C-4 Cl by the sulfur of NAC was nonmutagenic, whereas the minor product, in which the C-3 Cl was displaced, was significantly less mutagenic than MCA but still slightly mutagenic. Thus, the interaction of MX with glutathione and other cellular macromolecules may substantially reduce the risk through binding or altering MX before it can react with DNA.

On the other hand, the mutagenicity of MX may be potentiated by other common mutagens to which humans are exposed. In a recent study by Watanabe et al. (1997), the mutagenicity of MX in *E. coli* was enhanced in combination with the food mutagens Trp-P-1 and MeIQ. These heterocyclic amines, which are not mutagenic in the absence of metabolic activation, enhanced the mutagenicity of MX, even without S-9 mix. Thus, the effects of MX (and presumably other MX-related compounds) in vivo are quite complex, and the combined effects of other mutagens, as well as inactivation by cellular macromolecules, must be considered for risk evaluation.

Alternative Disinfection Processes

The mutagenicity in *Salmonella* of water samples derived from alternative water treatment procedures differs significantly, even given

comparable total organic carbon (TOC) concentrations in the source water (DeMarini et al., 1995; Smeds et al., 1997). Ozonation did not increase the mutagenicity of water relative to the source water, whereas disinfection with chlorine in any form increased the mutagenic potency of the water by 2-36 times (DeMarini et al., 1995). In the study by Smeds et al. (1997), pretreatment of surface water with ozone followed by chloramination yielded water samples with the lowest mutagenic potency, whereas water samples in which chlorine was used for pretreatment and as a postdisinfectant were among the most mutagenic. Likewise, MX was detected in the lowest concentrations in water samples in which either ozone was used as a preoxidant or no preoxidant was used in disinfection of surface waters.

Interest in the United States in ozonation as an alternative to chlorination of drinking water began to grow in the early 1970s as a result of the realization that the traditional disinfection process involving free residual chlorine (HOCl) produced a host of trihalomethanes believed to have undesirable health effects (White, 1999c). In addition to meeting U.S. federal requirements for removal or inactivation of microbiological contaminants (e.g., the 1989 Safe Water Treatment Rule), water utilities are faced with the task of producing water that meets the aesthetic requirements of the consumer, including taste, smell, and appearance. Ozonation has emerged as a candidate process that will allow utilities to meet these requirements. When ozone is used alone as the sole oxidant in a water treatment process, its reactions are relatively slow and selective. However, when used in conjunction with hydrogen peroxide, the ozone molecule is decomposed to a hydroxyl free radical that

serves as a more potent and less selective oxidant than ozone alone (White, 1999c). It is this hydroxy free radical that is solely responsible for the destruction of phenolic tastes in surface waters.

Some of the typical disinfection by-products produced by ozonation are formaldehyde, acetaldehyde, acetone, and carboxylic acids. However, ozonation of source water containing high concentrations of bromide ions produces a number of brominated disinfection by-products such as bromoform, dibromoacetic acid, cyanogen bromide, and inorganic bromate. A range of adverse health effects has been associated with various brominated DBPs, including sister chromatid exchanges, DNA damage in *E. coli* PQ37, and mutagenicity in *Salmonella* TA100 (Giller et al., 1997; Landi et al., 1999). Bromate induces gene mutations at the *Hprt* locus and chromosome aberrations in V79 Chinese hamster cells (Speit et al., 1999), increases cell proliferation in the proximal tubules of male rats (Umemura et al., 1993), and is carcinogenic in rat kidney, thyroid, and mesothelium and in the kidney of the male mouse (De Angelo et al., 1998). Thus, potentially harmful DBPs are associated both with disinfection using free chlorine and by ozonation.

Among the strategies that might be used to remove DBPs after formation is the use of a biological filtration system where biodegradation of organic compounds will occur. If secondary disinfection by chlorination following ozonation takes place after filtration, a biofilm can develop on the filter medium capable of degrading formaldehyde, assimilable organic carbon (AOC, which is the fraction of dissolved organic carbon that is degradable by bacteria), and other ozonation by-products. Ozone, used in combination with either UV light,

hydrogen peroxide, or elevated pH can be used to oxidize volatile organic compounds including trichloroethylene, carbon tetrachloride, phenols, acetic and oxalic acids, and certain pesticides (White, 1996c). A necessary aspect of this strategy is the determination of proper biological techniques that will assure complete mineralization of the AOC within the treatment plant. Any remaining AOC becomes a source of nutrition for microorganisms in the distribution system. Without complete mineralization, a post-disinfection step must be added. Given the inconclusive evidence of human cancer risk from disinfection by-products of chlorination (including MX and MCF) and the potentially equal or greater health risks from brominated DBPs formed during ozonation of raw water containing high levels of bromide ions, it may be difficult to justify the cost of developing and implementing new technology involved in disinfection of drinking water by ozonation.

Consumption of mutagenic drinking water, i.e., from a municipality using chlorinated surface water, has been associated with an increase in kidney and bladder cancer (Koivusalo et al., 1994). However, no oncogene or tumor suppressor gene has been found to be mutated in the majority of human bladder tumors (Reznikoff et al., 1993). Only 34% of human bladder tumors have been found to have p53 mutations, and among these 50% are GC → AT transitions at GpC sites, which are likely to arise spontaneously from the deamination of cytosine at these sites (Greenblatt et al., 1994). Given the results of this and other studies (DeMarini et al., 1995; Hyttinen et al., 1995, 1996; Knasmüller et al., 1996; Watanabe-Akanuma and Ohta, 1994), one would predict a predominance of GC → TA transversions in target genes in tumors associated with consumption of

chlorinated drinking water.

MX, MCF, and related chlorohydroxyfuranones comprise only one class among a number of halogenated disinfection by-products that pose potential risks to human health. Compared to estimated trihalomethane concentrations in drinking water of 100 parts per billion (based on the EPA's current maximum contaminant level), levels of MX have been reported to be 3 to 67 parts per trillion. Thus, trihalomethanes may be present in drinking water in levels 1000 times greater than MX. Melnick et al. (1997) have estimated the cancer potency of MX, based on the induction of cholangiomas in the livers of MX-treated rats (Komulainen et al., 1997), to be 100% per mg MX per kg body weight per day. In contrast, estimates for chloroform-induced kidney tumors in rats are in the range of 0.6% per mg/kg/day (100%/4.6 nanomoles MX/kg/day versus 0.6%/8.4 nanomoles chloroform/kg/day). In other words, the cancer potency of MX is approximately 170 times greater than chloroform. When cancer potency and exposure are combined, the potential risks from MX may be about one-tenth those of chloroform and other trihalomethanes.

Future Directions

MX remains an important subject of research. The actual DNA lesions from interaction with MX have yet to be determined, especially the lesions that are processed into complex frameshift mutations. The basis for the predominance of GC versus AT sites of mutations is unclear. It is presumably due to the sites of damage, the nature of which is unknown. In terms of adduct formation, MX provides an example of a highly reactive electrophile that may interact with DNA through a number of mechanisms,

including formation of adducts at AT sites and a one-electron reduction of MX at GC sites followed by depurination (Tuppurainen, 1997). Studies of health effects of less reactive MX-related compounds such as CMCF and brominated analogues of MX are also important; such compounds may be less likely to react with proteins or carbohydrates than with nucleic acids and, thus, may have a greater chance of reacting with DNA than MX. The basis for mutagenic potency differences between MX and MCF is also unknown. Whatever the actual lesions are, the mutagenic efficiencies are clearly different.

An overall assessment of the total mutagenicity of drinking water is still needed. The XAD extracts of drinking water prepared from different source waters and by different disinfection procedures have been studied to a limited extent in *Salmonella* and to an even more limited extent in cell cultures in order to determine chromosomal mutations. The genotoxicity of the volatile fraction of drinking water as a mixture has never been studied; methods for this type of study still need to be developed. Finally, the assessments of mutagenicity from both XAD extracts and volatile mixtures must be combined to produce an overall assessment of the mutagenicity of drinking water.

Beyond mechanistic toxicology studies, further engineering efforts may lead to cost-effective methods of producing microbiologically safe, aesthetically pleasing drinking water that poses even less risk to human health than does most drinking water produced by standard chlorination methods.

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