

Pasi Hakulinen

# Experimental Studies on Cellular Mechanisms of the Carcinogenicity of 3-Chloro-4-(Dichloromethyl)-5-Hydroxy-2(5H)-Furanone (MX)

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National Public Health Institute Kuopio, Finland

**Pasi Hakulinen**

EXPERIMENTAL STUDIES ON CELLULAR  
MECHANISMS OF THE CARCINOGENICITY OF 3-  
CHLORO-4-(DICHLOROMETHYL)-5-HYDROXY-  
2(5*H*)-FURANONE (MX)

ACADEMIC DISSERTATION

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**Kannen kuva – cover graphic: The transfer of Lucifer yellow dye into contiguous cells through gap junctions. Left: untreated cells, right: MX-exposed cells.**

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## ABSTRACT

Several different disinfection by-products (DBPs) are formed during the chlorination of raw water. The DBPs which pose a cancer risk to man are not known. One candidate group is the chlorohydroxyfuranones (CHF). Of the CHFs, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX), 3-chloro-4-(chloromethyl)-5-hydroxy-2(5H)-furanone (CMCF), 3,4-dichloro-5-hydroxy-2(5H)-furanone (MCA), and 3-chloro-4-methyl-5-hydroxy-2(5H)-furanone (MCF) are all genotoxic in mammalian cells *in vitro*. MX is also carcinogenic in Wistar rats.

This thesis focuses on the underlying cellular mechanisms involved in MX-induced carcinogenicity. Point mutations were analyzed in the *p53* tumor suppressor gene (exons 4-7) and the *Ki-*, *Ha-*, and *N-ras* oncogenes (exons 1-2) of the liver tumors of the MX carcinogenicity study in rats. The expression of *p53* protein was examined immunohistochemically in the liver and thyroid gland tumors. In addition, the expression of *p21* *Ki-ras* protein was determined in several thyroid gland tumors.

No mutations were detected in *ras* genes in 50 rat liver tumors. The mutation frequency of *p53* gene was low (4 mutations). Moreover, the mutations had no consistent pattern, scattering to different codons and positions of the codon. Immunohistochemical analyses revealed that hepatocellular adenomas and carcinomas did not overexpress *p53* protein. Instead, all the cholangiomas and cholangiocarcinomas, which originate from the bile duct epithelial cells, overexpressed *p53* protein. The epithelial cells of the hyperplastic bile ducts of aged rats overexpressed *p53* protein independently of the MX treatment. The MX-induced thyroid tumors did not have an abnormal expression of *p53* or *p21* *Ki-ras* proteins.

In other experiments, the effects of MX, CMCF, MCA, and MCF were investigated on gap junctional intercellular communication (GJIC), first in Balb/c 3T3 mouse fibroblast cells and then in WB-F344 rat liver epithelial cells, the target cells of MX tumorigenicity. Inhibition of GJIC is a common mechanism among tumor promoters. Concentration- and time-responses for inhibition of GJIC by CHF were studied in both cell lines. In addition, the role of protein kinase C (PKC) and

mitogen-activated protein kinase (MAPK) signaling pathways in the inhibition of GJIC by CHF<sub>s</sub> was defined in WB-F344 cells. The effects of CHF<sub>s</sub> on the gap junction structural protein, connexin43 (Cx43), and its mRNA levels were also determined.

All the studied CHF<sub>s</sub> inhibited GJIC dose-dependently in both cell lines. The orders of potency to inhibit GJIC in Balb/c 3T3 and WB-F344 cells were MX>MCA>CMCF>MCF and MX>CMCF≈MCA>MCF, respectively. In Balb/c 3T3 cells MX inhibited GJIC already at nanomolar concentrations. In WB-F344 cells, the inhibition of GJIC by CHF<sub>s</sub> was reversible and was dependent upon the activation of the MAPK signaling pathway. CHF<sub>s</sub> decreased the expression of the Cx43 protein without altering its mRNA level. These results suggest that the main mechanism by which CHF<sub>s</sub> inhibit GJIC in the liver cells was to decrease the expression of Cx43.

Altogether, the results suggest that point mutations in *p53* and *ras* genes do not contribute to the MX-induced liver and thyroid tumorigenesis in rats. In contrast, inhibition of GJIC may be one mechanism by which MX can promote tumor development.

Keywords: chlorinated drinking water, chlorohydroxyfuranones, MX, carcinogenesis, rat, ras, p53, gap junctional intercellular communication, GJIC, Balb/c 3T3, WB-F344

Pasi Hakulinen, Kokeellisia tutkimuksia 3-kloori-4-(dikloorimetyyli)-5-hydroksi-2(5*H*)-furanonin (MX) karsinogeenisuuden solutason mekanismeista.

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## TIIVISTELMÄ

Juomaveden kloorauksessa muodostuu useita erilaisia sivutuotteita. Ei kuitenkaan tiedetä, mitkä sivutuotteet aiheuttavat syöpäriskin. Yksi tällainen mahdollinen sivutuoteryhmä on kloorihydroksifuranonit, joista 3-kloori-4-(dikloorimetyyli)-5-hydroksi-2(5*H*)-furanoni (MX), 3-kloori-4-(kloorimetyyli)-5-hydroksi-2(5*H*)-furanoni (CMCF), 3,4-dikloori-5-hydroksi-2(5*H*)-furanoni (MCA) ja 3-kloori-4-metyyli-5-hydroksi-2(5*H*)-furanoni (MCF) ovat genotoksisia nisäkässoluissa *in vitro*. MX aiheuttaa myös kasvaimia Wistar-rotissa.

Tässä väitöskirjatutkimuksessa on selvitetty MX:n karsinogeenisuuden solutason mekanismeja. Rotilla tehdyn MX:n syöpäkokeen maksakasvaimista analysoitiin pistemutaatioita *p53* tuumorisuppressorigeenissä (eksonit 4-7) ja *Ki-*, *Ha-* ja *N-ras* onkogeneissä (eksonit 1-2). *p53*-proteiinin ilmentymistä tutkittiin immunohistokemiallisesti maksa- ja kilpirauhaskasvaimista. Lisäksi määritettiin *p21* *Ki-ras*-proteiinin ilmentyminen useista kilpirauhaskasvaimista.

50:ssä tutkitussa rotan maksakasvaimessa ei havaittu lainkaan mutaatioita *ras*-geeneissä. Mutaatiofrekvenssi *p53*-geenissä oli alhainen (4 mutaatiota). Mutaatioilla ei ollut myöskään yhteneväisyyttä. Ne jakaantuivat eri kodoneihin ja eri paikkoihin kodoneissa. Immunohistokemialliset tutkimukset paljastivat, että hepatosellulaariset adenoomat ja karsinoomat eivät ilmentäneet *p53*-proteiinia poikkeavasti. Sen sijaan kaikki kolangioomat ja kolangiokarsinoomat, jotka ovat peräisin sappiteistä, ilmensivät *p53*-proteiinia. Vanhempien rottien sappiteiden epiteelisolut ilmensivät *p53*-proteiinia riippumatta MX-käsittelystä. MX:n aiheuttamissa kilpirauhaskasvaimissa ei esiintynyt poikkeavaa *p53*- tai *p21* *Ki-ras*-proteiinin ilmentymistä.

Tutkittiin myös MX:n, CMCF:n, MCA:n ja MCF:n vaikutuksia solujen väliseen kommunikointiin. Tutkimukset aloitettiin hiiren Balb/c 3T3 fibroblastisoluilla ja jatkettiin rotan WB-F344 maksaepiteelisoluilla, jotka ovat MX:n karsinogeenisuuden kohdesoluja. Solujen aukkoliitosten kautta tapahtuva solujen välisen kommunikaation esto on yleinen tuumoripromoottorien mekanismi. Molemmilla solulinjoilla määritettiin pitoisuus- ja aikavasteet

kloorihydroksifuranonien aiheuttamalle solu-solu kommunikaation estymiselle. Lisäksi WB-F344-soluilla selvitettiin proteiinikinaasi C:n (PKC) ja mitogeenin aktivoimien proteiinikinaasien (MAPK) merkitystä kloorihydroksifuranonien tässä vaikutuksessa. Kloorihydroksifuranonien vaikutukset aukkoliitosten rakenneproteiiniin, konneksiini43:een (Cx43), ja sen mRNA:han määritettiin myös.

Kaikki tutkitut kloorihydroksifuranonit estivät aukkoliitosten kautta tapahtuvaa solujen välistä kommunikointia annosvasteisesti molemmissa solulinjoissa; Balb/c 3T3-soluissa järjestyksessä MX>MCA>CMCF>MCF ja WB-F344-soluissa MX>CMCF≈MCA>MCF. Balb/c 3T3-soluissa MX esti solu-solu kommunikaatiota jo nanomolaarisilla pitoisuuksilla. WB-F344-soluissa kloorihydroksifuranonien aiheuttama solu-solu kommunikaation estyminen oli palautuvaa ja riippui MAPK-signaaliketjun aktivoitumisesta. Kloorihydroksifuranonit vähensivät Cx43:n ilmentymistä muuttamatta kuitenkaan sen mRNA-tasoa. Näiden tulosten mukaan Cx43:n ilmentymisen vähentäminen oli päämekanismi, jolla kloorihydroksifuranonit estävät solu-solu kommunikaatiota maksasoluissa.

Yhteenvetona voidaan todeta, että MX:n aiheuttamat maksa- ja kilpirauhaskasvaimet rotissa eivät johdu pistemutaatioista *p53*- ja *ras*-geeneissä. Sitä vastoin solu-solu kommunikaation estyminen voi olla yksi mekanismi, jolla MX edistää kasvainten kehittymistä.

Avainsanat: kloorattu juomavesi, kloorihydroksifuranonit, MX, karsinogeneesi, rotta, ras, p53, solu-solu kommunikaatio, GJIC, Balb/c 3T3, WB-F344

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## ABBREVIATIONS

AP site	Apurinic/apyrimidinic site
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
CA	Chromosome aberration
CHF	Chlorohydroxyfuranone
CHO cells	Chinese hamster ovary cells
CMCF	3-Chloro-4-(chloromethyl)-5-hydroxy-2(5 <i>H</i> )-furanone
Cx	Connexin protein (gap junction structural protein)
DBP	Disinfection by-product
DHPN	<i>N</i> -bis(2-hydroxypropyl)nitrosamine
DNA	Deoxyribonucleic acid
EMX	( <i>E</i> )-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid
GJC	Gap junctional intercellular communication
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GSH	Glutathione
<i>hprt</i> gene	Hypoxanthine phosphoribosyl transferase gene
IARC	International Agency for Research on Cancer of the World Health Organization (WHO)
LUMO	Lowest unoccupied molecular orbital
MAPK	Mitogen-activated protein kinase
MC	3-Methylcholanthrene
MCA	3,4-Dichloro-5-hydroxy-2(5 <i>H</i> )-furanone
MCF	3-Chloro-4-methyl-5-hydroxy-2(5 <i>H</i> )-furanone
MEK 1	Mitogen-activated protein kinase kinase (upstream activator kinase of the MAPK)

MN	Micronuclei
MNNG	<i>N</i> -methyl- <i>N'</i> -nitro- <i>N</i> -nitrosoguanidine
mRNA	Messenger RNA
MX	3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5 <i>H</i> )-furanone
NBT	Nitroblue tetrazolium
PCR	Polymerase chain reaction
PKC	Protein kinase C
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SCE	Sister chromatid exchange
SDS-PAGE	Sodium dodecylsulphate – polyacrylamide gel electrophoresis
SL/DT assay	Scrape-loading dye transfer assay
SSCP	Single-strand conformation polymorphism
TBDE test	Trypan blue dye exclusion test
TCDD	2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin
TGGE	Temperature gradient gel electrophoresis
TPA	12- <i>O</i> -tetradecanoylphorbol-13-acetate
TSH	Thyroid stimulating hormone
UDS	Unscheduled DNA synthesis
z-MX	Z-2-chloro-3-(dichloromethyl)-4-oxo-butenic acid

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

- I** Komulainen, H., Hakulinen, P., Servomaa, K., Makkonen, K., Vasara, R., Mäki-Paakkanen, J., Kosma, V.-M., 2000. No consistent pattern of mutations in *p53* and *ras* genes in liver tumors of rat treated with the drinking water mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX). *Environ. Mol. Mutagen.* 36, 292-300.
- II** Hakulinen, P., Kosma, V.-M., Komulainen, H., 2002. Expression of p53 and p21 Ki-ras proteins in rat thyroid gland tumors induced by 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX). *Anticancer Res.* 22, 703-706.
- III** Hakulinen, P., Mäki-Paakkanen, J., Naarala, J., Kronberg, L., Komulainen, H., 2004. Potent inhibition of gap junctional intercellular communication by 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) in BALB/c 3T3 cells. *Toxicol. Lett.* 151, 439-449.
- IV** Hakulinen, P., Rintala, E., Mäki-Paakkanen, J., Komulainen, H., 2005. Altered expression of connexin43 in the inhibition of gap junctional intercellular communication by chlorohydroxyfuranones in WB-F344 rat liver epithelial cells. *Toxicol. Appl. Pharmacol.* In press (*published online*).

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# 1 INTRODUCTION

The treatment of drinking water for safe use was one of the most significant achievements of the last century in public health. Chlorination of drinking water reduced the mortality rates associated with waterborne pathogens and chlorination is still the most common disinfection technique worldwide. It is easily applied, controlled, and monitored. Chlorine is a cheap and highly effective disinfectant against most known pathogens. However, chlorination suffers from some serious drawbacks. One major disadvantage is the formation of disinfection by-products (DBPs). DBPs are formed during the chlorination process in the waterworks when chlorine reacts with natural organic matter present in raw water. The actual levels of DBPs can vary depending on the amount of chlorine added and the quality of the raw water. Chlorinated water prepared from surface waters contains substantially higher levels of DBPs than chlorinated water from ground waters. This is because the content of organic matter is lower in ground waters (IARC, 1991).

The detection of by-products in chlorinated drinking water has raised concern over the potential health risks of long-term exposure to DBPs. Chlorination may account for a substantial portion of the cancer risk associated with drinking water. Several epidemiological studies point to an association between the consumption of chlorinated drinking water and an increased risk of cancers of the bladder, rectum, pancreas, kidney, stomach, colon and lymphomas (Cantor *et al.*, 1987; Morris *et al.*, 1992; 1995; Koivusalo *et al.*, 1994; 1995; 1997; Doyle *et al.*, 1997; Villanueva *et al.*, 2004). Several hundreds of different by-products have been identified in the drinking water and there are likely to be more DBPs still to be identified. Many DBPs have mutagenic and carcinogenic properties, but the broad category of DBPs includes also compounds whose toxicological effects have not yet been evaluated (IARC, 1991; Richardson *et al.*, 2002).

The by-products or groups of by-products in chlorinated drinking water responsible for the cancer risk to man are not known. One candidate group is the chlorohydroxyfuranones (CHF<sub>s</sub>). Of the CHF<sub>s</sub>, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) is known to be an extremely potent, direct acting mutagen in *Salmonella typhimurium* strain TA100 (Meier *et al.*, 1987a) and a multisite carcinogen in Wistar rats (Komulainen *et al.*, 1997). In the overall evaluation conducted by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) MX was designated as a group 2B carcinogen (possibly carcinogenic to humans) (IARC, 2004).

In this thesis, the underlying cellular mechanisms of MX carcinogenicity in rats were elucidated to obtain further information for assessment of its role in the cancer risk. The effects of three other CHF's were evaluated on tumor promoting properties to elucidate also their possible role in the cancer risk. The MX-induced tumors in rats were the starting material for the present work. Mutations in *ras* and *p53* genes were believed to be important candidates in tumor development. The work was expanded to tumor promotion studies, due to observation that MX promoted the development of the transformation foci in the two-stage mouse fibroblast cell transformation assay *in vitro* (Laaksonen *et al.*, 2001).

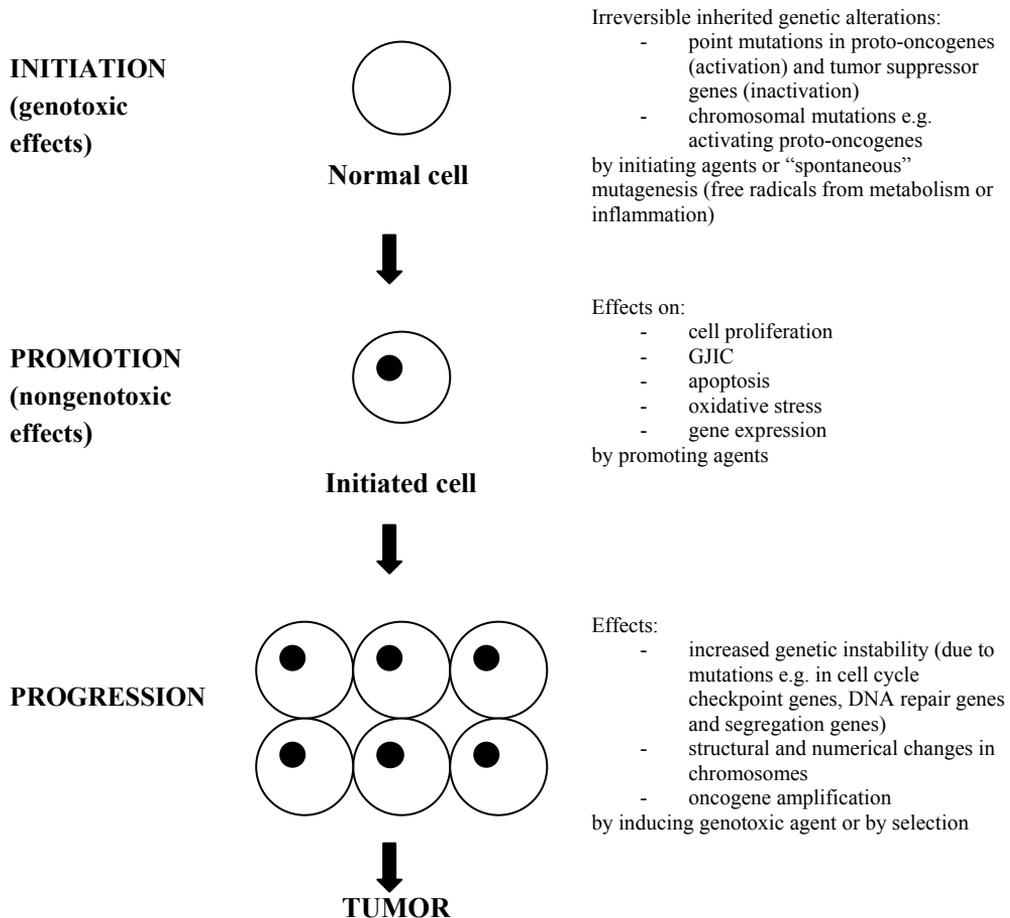
## 2 REVIEW OF THE LITERATURE

### 2.1 The process of chemical carcinogenesis

Cancer usually develops slowly, with a latent period between the initial exposure to a chemical carcinogen and the ultimate development of malignant neoplasia. As normal cells progress to the neoplastic stage, multiple genetic and epigenetic changes occur. The general multistage concept of chemical carcinogenesis consists of three individual stages: initiation, promotion and progression stages (Barrett, 1993; Pitot, 1993) (Fig. 1). This may be oversimplification, carcinogenesis is a very complex process involving an array of effects and mechanisms (Bertram, 2001). Many carcinogens have both genetic and epigenetic properties and their primary mechanism of action can vary depending on the target tissue. Thus even the three-stage model of initiation, promotion and progression cannot describe the carcinogenic process adequately (Barrett, 1993).

#### 2.1.1 Initiation

Initiation is the process in which genotoxic agents evoke irreversible changes in the DNA resulting in altered cells with a clonal expansion advantage. Often the initiators are chemically-reactive xenobiotics, which are metabolized to activated forms which in turn produce carcinogen-DNA adducts. If these DNA adducts can evade the cellular repair mechanisms and persist, this may lead to miscoding, resulting in permanent mutations. In the carcinogenesis process, two classes of genes are the critical targets of genotoxic agents. Genes which are activated by a point mutation are called oncogenes (e.g. *ras*) and genes which are inactivated by a point mutation are called tumor suppressor genes (e.g. *p53*) (Bertram, 2001). Each cell contains two alternate forms of each gene, alleles. Activation of a single allele of an oncogene is sufficient to induce some aspects of the neoplastic phenotype even though there may be the simultaneous expression of the other normal allele. Because in most cases the normal tumor suppressor allele can function in the presence of the damaged allele, both copies must be inactivated before loss of function is manifested. Genotoxic agents can influence the activation of oncogenes by inducing point mutations, chromosome rearrangements and gene amplification. Loss of function of tumor suppressor genes can be a consequence of point mutations, deletions and chromosome loss (Diamandis, 1997; Bertram, 2001).



**Figure 1. The general multistage model of chemical carcinogenesis (modified from Klaunig *et al.*, 2000). Initiation:** Initiating agents cause irreversible mutations in the genome. **Promotion:** Promoting agents cause the expansion of initiated cell clones. **Progression:** Preneoplastic cells progress into a malignant cell population.

#### 2.1.1.1 *p53* tumor suppressor gene

The *p53* tumor suppressor gene is mutated in the majority of human cancers (Hainaut and Hollstein, 2000), but clearly less frequently in tumors induced in animals (Stanley, 1995; Perantoni and Rice, 1999). The timing when mutations in the *p53* gene occur during cancer development is variable from one cancer to

another. *p53* mutations seem to occur at an early stage in many types of cancer, which are directly caused by exogenous carcinogens (Guimaraes and Hainaut, 2002). The *p53* gene encodes for p53 protein that participates in many cellular functions: cell cycle control, DNA repair and programmed cell death (apoptosis). The p53 protein is a sensor of multiple forms of genotoxic (e.g. radiation, carcinogen) and non-genotoxic stress (e.g. hypoxia) (Pluquet and Hainaut, 2001). In response to cellular stress, p53 protein can induce cell cycle arrest which allows the cells to repair DNA damage before further rounds of replication. If the DNA damage is excessive, p53 can induce apoptotic cell death. Thus, p53 can prevent the growth and impair the survival of potentially malignant cells. If the point mutations in the *p53* gene induce the inactivation of the p53 protein function, then the cell cycle progresses into replication despite the presence of DNA damage (Ryan *et al.*, 2001).

#### 2.1.1.2 *Ras* oncogenes

*Ras* oncogenes (Ki-, Ha-, and N-*ras*) are frequently mutated in human cancers and rodent tumors (Bos, 1989; Stanley, 1995; Sills *et al.*, 1999; Barletta *et al.*, 2004). *Ras* genes encode for 21-kDa proteins (p21ras) which are associated with the inner face of the plasma membrane and have guanosine triphosphate/guanosine diphosphate (GTP/GDP) binding activity (Barbacid, 1987). Most ras molecules in the cell exist in their inactive GDP-bound state. In normal cells, the hydrolysis of GTP to GDP returns the active ras protein to the inactive state. The *ras* mutations found in tumors lock the ras protein in a constitutively active GTP-bound state (Singh *et al.*, 2005). Ras proteins are activated in response to a wide variety of extracellular stimuli (e.g. chemical agents, growth factors, cytokines, adhesion signals) and regulate downstream signaling pathways (e.g. ras/raf/MEK/MAPK). The mutated forms of ras protein are known to stimulate proliferation, transformation and cell differentiation. In physiological conditions, ras proteins function as intracellular switches in the signaling pathways that control multiple biological processes including cell cycle progression and apoptosis (Macaluso *et al.*, 2002; Rodriguez-Viciano *et al.*, 2004).

#### 2.1.2 Promotion

Promotion is the stage when the clonal proliferation of initiated cells occurs in the cancer process (Fig. 1). In this stage, the promoting agents modulate cell growth and cell death without inducing mutations. In contrast to the initiators, the effect of promoters is reversible and exhibits a threshold. The exact mechanisms by which promoters cause their effects have not been established, but several mechanisms

may be involved in nongenotoxic chemical carcinogenesis e.g. induction of cell proliferation in the target tissue, inhibition of cell-to-cell communication, blockade of apoptosis, production of reactive oxygen species (ROS), and modification of gene expression (Klaunig *et al.*, 2000).

#### 2.1.2.1 Gap junctional intercellular communication (GJIC)

The reversible inhibition of GJIC has been demonstrated to be a common effect of many chemical tumor promoters (Trosko and Chang, 1988). Gap junctions, composed of connexin (Cx) proteins, are membrane-associated channels, which permit the direct intercellular exchange of low molecular weight (<1-2 kDa) molecules (e.g. sodium, potassium, calcium, cAMP, ATP, inositol triphosphate) between the cytoplasm of adjacent cells (Lawrence *et al.*, 1978; Pitts and Finbow, 1986; Saez *et al.*, 1989). This has a major physiological role of intercellular communication to maintain homeostatic control of growth, development, differentiation, apoptosis, and adaptive responses of differentiated cells in solid tissues (Trosko and Ruch, 1998). Extra-cellular signals, such as tumor promoting chemicals, can induce changes in gap junction function by causing transcriptional, translational or post-translational modifications of the Cxs (Trosko and Ruch, 1998; Chipman *et al.*, 2003). Therefore, disruption of GJIC results in malfunction in cell growth and loss of homeostatic control and could ultimately be a cause of promotion of tumors (Trosko *et al.*, 1998).

### 2.1.3 Progression

In the progression of carcinogenesis, the initiated cells progress into forming a malignant cell population (Fig. 1). The stage of progression is characterized by increasing aggressiveness of the tumor. The major hallmark of the stage of progression is evolving genetic instability. This is manifested both at the single nucleotide level, resulting in accumulation of multiple point mutations, or at the chromosomal level where it is reflected in translocations, deletions, amplifications and whole chromosome aneuploidy (Loeb and Loeb, 2000; Pitot, 2001).

The findings of MX and three other CHF's [3-chloro-4-(chloromethyl)-5-hydroxy-2(5*H*)-furanone (CMCF), 3,4-dichloro-5-hydroxy-2(5*H*)-furanone (MCA), and 3-chloro-4-methyl-5-hydroxy-2(5*H*)-furanone (MCF)] that are likely to contribute to the chemical carcinogenesis are discussed in the following paragraphs.

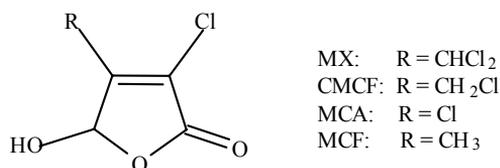
## 2.2 Occurrence of chlorohydroxyfuranones (CHF<sub>s</sub>)

CHF<sub>s</sub> are formed during disinfection treatment of drinking water as by-products from the reactions of chlorine with humic acid materials present in surface waters (Hemming *et al.*, 1986; Kronberg and Franzén, 1993). MX has been detected in chlorinated drinking water all around the world e.g. in Australia, Canada, China, Finland, Japan, the Netherlands, Russia, Spain, the United Kingdom, and the United States (Kronberg and Vartiainen, 1988; Kronberg *et al.*, 1988; Backlund *et al.*, 1989b; Andrews *et al.*, 1990; Fawell and Horth, 1990; Suzuki and Nakanishi, 1990; Kinae *et al.*, 1992; Zou *et al.*, 1995; Romero *et al.*, 1997; Simpson and Hayes, 1998; US EPA, 2002; Wright *et al.*, 2002; Egorov *et al.*, 2003). Its concentration in tap water has varied from non-detectable levels up to 310 ng/l. The concentration of MX in chlorinated drinking water depends on the disinfection conditions. Formation of MX is favoured under acidic conditions and high chlorine doses (Backlund *et al.*, 1989a).

With respect to the other CHF<sub>s</sub>, the concentrations of CMCF, MCA, and MCF in chlorine-disinfected drinking waters in Finland do not differ notably from that of MX (Kronberg and Franzén, 1993; Smeds *et al.*, 1997). However, MX dominates as a contributor of the overall mutagenicity measured in *S. typhimurium* strain TA100. According to Kronberg and Franzén (1993) MX accounted for 43% but CMCF a mere 3%, of the total mutagenicity of chlorinated drinking water in the Ames test (strain TA100). The activity contribution of MCA and MCF was negligible. In another study, MX was observed to account on average for 31% (from 7 to 67%) of the total mutagenicity of the Finnish chlorinated drinking water extracts, while the total contribution of CMCF, MCA, and MCF was on average only 4% (Smeds *et al.*, 1997). It should, however, be noted that *S. typhimurium* TA100 is exceptionally sensitive to the mutagenicity of MX, this being in some way related to the properties of the strain and the structure activity characteristics of the compound.

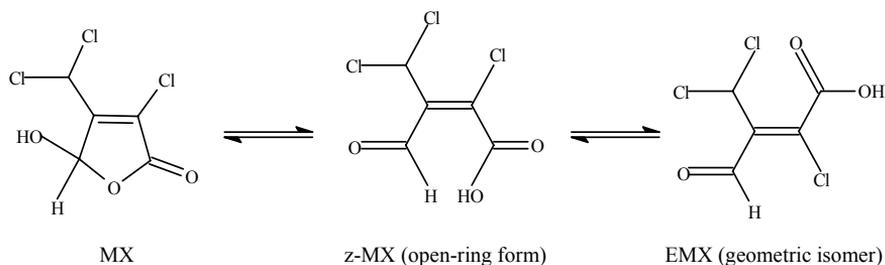
## 2.3 Chemical properties of chlorohydroxyfuranones (CHF<sub>s</sub>)

The chemical structures of MX, CMCF, MCA, and MCF are presented in Figure 2.



**Figure 2.** The chemical structures of the studied CHF.

MX undergoes a pH-dependent tautomerism. In acidic solutions, MX is a furanone but at physiological pH it is predominantly in the open-ring form (z-MX) (Meier *et al.*, 1987b; Holmbom *et al.*, 1989) (Fig. 3). However, the closed-ring form may be required for the cell membrane penetration and it may be a more active mutagen. The mutagenicity of MX in *S. typhimurium* strain TA100 has been noted to be about 3.3-fold higher at pH 6 compared to pH 8 (Meier *et al.*, 1987a). At pH 5-9, the isomerization to the geometric isomer of the open-ring form (EMX) takes place (Holmbom *et al.*, 1989). The EMX appears to have less than one-tenth of the mutagenic activity of MX (Kronberg *et al.*, 1988).



**Figure 3.** Tautomeric forms of MX. z-MX, Z-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid; EMX, (E)-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid.

MX is a polar, water soluble compound. pH has only a minor effect on the solubility of MX, but a major effect on its stability in water (Vartiainen *et al.*, 1991). MX has a low bioaccumulation property. The n-octanol/water distribution coefficient  $\log P_{ow}$  at the pH 2 is about 1 (Holmbom *et al.*, 1984; Vartiainen *et al.*, 1991) whereas at pH 9 it is about -1 (Vartiainen *et al.*, 1991).

Compared to MX, there is less data on the chemical properties of CMCF, MCA, and MCF. MCA has, like MX, the pH-dependent tautomerism. The furanone ring opens above pH 7. MCA is also more active in its closed form. The mutagenic activity at

pH 6 is about 10 times that observed at pH 8 (Meier *et al.*, 1986). The chemical half-life values ( $t_{1/2}$ ) for MX and CMCF, indicating stability under *S. typhimurium* mutagenicity assay conditions (about at pH 7), has been 11 and 44 hours, respectively (LaLonde *et al.*, 1991b).

## 2.4 Genotoxicity of chlorohydroxyfuranones (CHF<sub>s</sub>)

### 2.4.1 Genotoxicity *in vitro*

#### 2.4.1.1 MX

MX is genotoxic in various bacterial strains (IARC, 1991; McDonald and Komulainen, 2005). MX is a direct-acting mutagen, in fact the addition of a metabolic activator (S9 fraction of rat liver or human placental homogenate) decreases the bacterial mutagenicity of MX (Ishiguro *et al.*, 1987; Meier *et al.*, 1987a; Vartiainen *et al.*, 1989; Tikkanen and Kronberg, 1990; Cozzie *et al.*, 1993). MX is one of the most potent compounds ever tested in *S. typhimurium* strain TA100 (up to 13 000 revertants/nmol) (Meier *et al.*, 1987a), *S. typhimurium* strain TM677 and the *Escherichia coli* prophage-induction assay (DeMarini *et al.*, 1995). MX has high affinity for protein and other cellular nucleophiles, which decreases the mutagenic response of MX in bacteria. This kind of a decrease has been observed with bovine serum albumin (BSA), glutathione (GSH), SO<sub>2</sub>, S<sub>2</sub>O<sub>3</sub><sup>2-</sup>, pyrrolidine, cysteine, cysteamine, dithiothreitol, and 2-mercaptoethanol (Ishiguro *et al.*, 1987; Haataja *et al.*, 1991; Cozzie *et al.*, 1993; Watanabe *et al.*, 1994).

MX is genotoxic also in mammalian cells; MX-induced DNA lesions, unscheduled DNA synthesis (UDS), gene mutations, sister chromatid exchanges (SCEs), chromosome aberrations (CAs) and micronuclei (MN) have been detected in a number of rat, mouse, hamster and human cell lines (Table 1).

**Table 1.** MX genotoxicity in mammalian cells *in vitro*.

<b>Endpoint/cell line</b>	<b>References (in alphabetical order)</b>
<b>DNA damage (alkaline elution, alkaline DNA unwinding assay, comet assay, unscheduled DNA synthesis)</b>  Chinese hamster ovary (CHO) cells (+) <sup>a</sup> Chinese hamster V79 cells (+) Mouse primary hepatocytes (+) Rat primary hepatocytes (+) Rat primary testicular cells (+) Rat testicular germ cells (+) Human lymphoblastoid cell line (CCRF-CEM) (+) Human peripheral blood mononuclear cells (PBMC) (+) Human proximal tubular epithelial cells (LLC-PK <sub>1</sub> ) (+) Human white blood cells (+) Promyelocytic human leukaemia cell line (HL-60) (+)	Brunborg <i>et al.</i> , 1991; Chang <i>et al.</i> , 1991; Holme <i>et al.</i> , 1999; Le Curieux <i>et al.</i> , 1999; Marsteinstredet <i>et al.</i> , 1997a; Mäki-Paakkanen <i>et al.</i> , 2001; Nunn and Chipman, 1994; Nunn <i>et al.</i> , 1997
<b>Mutation (induction of TG<sup>r</sup> or Oua<sup>R</sup> mutants, or mutations at the thymidine kinase locus)</b>  CHO cells (+) Chinese hamster V79 cells (+,-) <sup>b</sup> L5178Y mouse lymphoma cells (+) Human B-lymphoblastoid cell lines (+)	Harrington-Brock <i>et al.</i> , 1995; Hyttinen <i>et al.</i> , 1996; Jansson and Hyttinen, 1994; Matsumura <i>et al.</i> , 1994; Mäki-Paakkanen <i>et al.</i> , 1994; Woodruff <i>et al.</i> , 2001
<b>SCEs</b>  CHO cells (+) Chinese hamster V79 cells (+) Rat peripheral lymphocytes (+)	Brunborg <i>et al.</i> , 1991; Jansson <i>et al.</i> , 1993; Mäki-Paakkanen <i>et al.</i> , 1994; 2001
<b>CAs</b>  CHO cells (+) L5178Y mouse lymphoma cells (+) Rat peripheral lymphocytes (+)	Harrington-Brock <i>et al.</i> , 1995; Jansson <i>et al.</i> , 1993; Meier <i>et al.</i> , 1987a; Mäki-Paakkanen <i>et al.</i> , 1994; 2001
<b>MN</b>  L5178Y mouse lymphoma cells (+)	Le Curieux <i>et al.</i> , 1999

<sup>a</sup>Positive result.

<sup>b</sup>Positive, when MX was diluted in a buffered salt solution, but negative when MX was diluted in tissue culture medium (Matsumura *et al.*, 1994).

In mammalian cells, the capability of GSH to reduce the mutagenic activity of MX does not seem to be so straightforward as in bacteria. In rat testicular cells, cellular GSH had only a minor effect on the MX-induced DNA damage (Brunborg *et al.*, 1991). In primary rat hepatocytes, MX did not produce detectable DNA strand breaks at similarly low concentrations as the mutagenicity in bacterial cells, which may indicate that there are more efficient protective mechanisms and structures in general in mammalian cells than in bacteria. Depletion of GSH did not enhance induction of DNA damage by MX in primary rat hepatocytes (Chang *et al.*, 1991).

MX can bind to purified DNA. MX induces single-strand DNA breaks and apurinic/apyrimidinic (AP) sites in bacteriophage PM2 and plasmid  $\Phi$ X174 DNA (Hyttinen and Jansson, 1995; LaLonde and Ramdayal, 1997). The presence of GSH increases the cleavage of  $\Phi$ X174 by MX (LaLonde and Ramdayal, 1997), although GSH decreases the bacterial mutagenicity of MX (Ishiguro *et al.*, 1987). According to the authors, that might be explained by the presence of GSH – MX reaction products that may be less potent bacterial mutagens, but better cleavage agents than MX (LaLonde and Ramdayal, 1997).

#### 2.4.1.2 CMCF, MCA, MCF

CMCF, MCA, and MCF, like MX, are direct-acting genotoxic compounds, but in bacteria they are less potent mutagens than MX. In general, the order of the potency of the four CHF's to induce *his* revertants in *S. typhimurium* strain TA100 is MX>CMCF>MCA>MCF (Ishiguro *et al.*, 1988; LaLonde *et al.*, 1991a; Kronberg and Franzén, 1993; Hyttinen *et al.*, 1995; Knasmüller *et al.*, 1996; Franzén *et al.*, 1998a; Mäki-Paakkanen *et al.*, 2004). Also in two *E. coli* K-12 strains that differ in their repair capacities [*uvrB/recA* (repair-deficient) vs. *uvr+/rec+* (repair-proficient)] the order of the genotoxic potency is MX>CMCF>MCA (Fekadu *et al.*, 1994). In *E. coli* strains, MX is approximately 5-fold more effective a genotoxin than CMCF and ten times more potent than MCA.

CMCF, MCA, and MCF induce DNA strand breaks, mutations, SCEs, and CAs in Chinese hamster ovary (CHO) cells (Jansson *et al.*, 1995; Niittykoski *et al.*, 1995; Mäki-Paakkanen *et al.*, 2001) and MN in L5178Y mouse lymphoma cells and UDS in primary hepatocytes of Fischer F344 rats (Le Curieux *et al.*, 1999). The order of the genotoxic potency of the CHF's differed in the various mammalian cell assays. In general, MCF has been the weakest genotoxin in the assays (2 – 200-fold weaker than MX). In the alkaline single cell gel, micronucleus, and UDS assay the genotoxic potencies of CMCF and MCA were comparable to or even higher than that of MX (Le Curieux *et al.*, 1999).

In plasmid  $\Phi$ X174, MCA evokes DNA strand cleavage at a higher rate than MX. In contrast to the situation with MX, the presence of GSH reduces the DNA cleavage action of MCA (LaLonde and Ramdayal, 1997).

## 2.4.2 Genotoxicity *in vivo*

### 2.4.2.1 MX

MX has genotoxic potential also *in vivo*, inducing DNA damage in a wide range of tissues of rats and mice (Table 2a). Increases in nuclear anomalies, including MN, have been observed in the duodenum of B6C3F1 mice after a single oral dose of MX (Daniel *et al.*, 1991) and MN are apparent in peripheral blood lymphocytes of Han/Wistar rats after oral administration of MX on three consecutive days (Mäki-Paakkanen and Jansson, 1995). Increases in SCEs have been observed in peripheral blood lymphocytes and kidney cells of Han/Wistar rats after administration to MX by gavage on three consecutive days, and in peripheral lymphocytes of rats exposed to MX by gavage for 14-18 weeks (Jansson *et al.*, 1993; Mäki-Paakkanen and Jansson, 1995). Other types of DNA damage (DNA single-strand breaks, alkali-labile sites, differential DNA repair) have been detected in a variety of tissues both in rats and mice after a single dose of MX (Furihata *et al.*, 1992; Fekadu *et al.*, 1994; Sasaki *et al.*, 1997).

MX-induced DNA damage has not been observed in all studies (Table 2b). MX does not appear to affect the bone marrow of rats and mice (Meier *et al.*, 1987a; Tikkanen and Kronberg, 1990; Brunborg *et al.*, 1991; Meier *et al.*, 1996; Sasaki *et al.*, 1997; Jansson, 1998). These observations have raised doubts about the genotoxicity of MX *in vivo*. First, MX is extremely potent only in bacteria. Second, in general it may be difficult to demonstrate genotoxicity *in vivo* due to the presence of several efficient prevention and correction mechanisms. For example, tissues seem to have different capacities to repair MX-induced DNA damage. Sasaki *et al.* (1997) found that a single oral dose of MX could induce DNA damage in mice, which persisted between 6-24 h in several tissues, though in liver, the effect reverted to background levels by three hours after administration. Therefore, timing in respect to dosing may be important for observation of the damage. The other reasons may be pharmacokinetics (distribution to tissues) and different cellular defence systems in addition to correction of DNA damage. Further evidence for *in vivo* genotoxicity of MX comes from studies with DNA-repair inhibition. Only mice pretreated with DNA repair inhibitors prior to MX administration, exhibited DNA damage in the liver, kidney, spleen, and colon (Holme *et al.*, 1999).

**Table 2a.** MX genotoxicity *in vivo* (positive results).

Species/strain	MX administration (effective doses in bold)	Endpoint	Tissue	Reference
<b>Mice</b>				
B6C3F1 (male)	oral gavage, single doses at 0.28, <b>0.37</b> , or <b>0.46</b> mmol/kg (24 h between dosing and sacrifice)	MN	duodenum	Daniel <i>et al.</i> , 1991
CD-1 (male)	oral gavage, single dose at <b>100</b> mg/kg (0, 1, 3, 6, 24 h)	DNA damage (comet assay)	liver, lung, kidney, brain, stomach, jejunum, ileum, colon, bladder	Sasaki <i>et al.</i> , 1997
Swiss Albino (male)	oral gavage, single doses at <b>4.3</b> , <b>13</b> , <b>40</b> , or <b>200</b> mg/kg (2 h)	DNA damage (animal-mediated assay)	stomach, lungs, intestine, liver, kidney, spleen	Fekadu <i>et al.</i> , 1994
<b>Rats</b>				
F344 (male)	oral gavage, single doses at 10, 20, or <b>48</b> mg/kg (2 h)	DNA single-strand breaks or alkali-labile sites	pyloric mucosa of the stomach	Furhata <i>et al.</i> , 1992
Han/Wistar (male)	oral gavage, three days at doses of 25, 50, <b>100</b> , or <b>150</b> mg/kg (2 h after the last dose)	SCEs, MN	peripheral blood lymphocytes, kidney cells	Mäki-Paakkanen and Jansson, 1995
Han/Wistar (male and female)	oral gavage, five days per week for 14 to 18 weeks at doses of <b>30</b> and <b>45-75</b> mg/kg (24-30 h after the last dose)	SCEs	peripheral blood lymphocytes	Jansson <i>et al.</i> , 1993

**Table 2b.** MX genotoxicity *in vivo* (negative results).

Species/strain	MX administration	Endpoint	Tissue	Reference
<b>Mice</b>				
NMRI (male and female)	intraperitoneal (i.p.), single dose at 4.4 or 8.8 mg/kg (24 h between dosing and sacrifice)	MN	bone marrow, polychromatic erythrocytes	Tikkanen and Kronberg, 1990
B6C3F1 (male)	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)	DNA damage (alkaline elution)	liver, kidney, spleen, colon	Holme <i>et al.</i> , 1999
Balb/c (male)	oral gavage, single dose at 100 mg/kg (3 or 16 h)	UDS	hepatocytes	Nunn <i>et al.</i> , 1997
CD-1 (male)	oral gavage, single dose at 100 mg/kg (0, 1, 3, 6, 24 h)	DNA damage (comet assay)	bone marrow	Sasaki <i>et al.</i> , 1997
Swiss-Webster (male and female)	oral gavage, two days at doses of 22.5, 45, or 90 mg/kg (sacrifice 48 or 72 h after the first dose)	MN	bone marrow	Meier <i>et al.</i> , 1987a
B6C3F1 (male and female)	oral gavage for 14 days at doses of 8, 16, 32, or 64 mg/kg (24 h)	MN	peripheral blood erythrocytes	Meier <i>et al.</i> , 1996
<i>gpt</i> delta C57BL/6J (male and female)	via drinking water at doses of 10, 30, or 100 ppm for 12 weeks	mutation (induction of <i>gpt</i> or <i>red/gam</i> mutants)	liver, lung	Nishikawa <i>et al.</i> , 2006
<b>Rats</b>				
Wistar (male)	oral gavage, single dose at 18, 63, or 125 mg/kg (1 h) or i.p., single dose at 18 mg/kg (1 h)	DNA damage (alkaline elution)	small and large intestine, stomach, liver, kidney, lung, bone marrow, urinary bladder, testis	Brunborg <i>et al.</i> , 1991
Wistar (male and female)	via drinking water for 104 weeks at doses of 5.9, 18.7, or 70 mg/l	MN	bone marrow, polychromatic erythrocytes	Jansson, 1998

#### 2.4.2.2 CMCF and MCA

Much less is known about the *in vivo* genotoxicity of other CHF's and the data do not allow any final conclusions to be drawn on this point. Nuclear anomalies have been detected in the duodenal, but not in the forestomach or proximal colon of B6C3F1 mice after administration of a single oral dose of 0.47 mmol/kg MCA (Daniel *et al.*, 1991). In the animal-mediated bacterial differential DNA repair assay (two *E. coli* K-12 strains as indicators of DNA damage in male Swiss Albino mice), CMCF and MCA have been observed to cause genotoxic effects in the bacteria isolated from several organs (stomach, lung, intestine, liver, kidney, spleen) after a single oral dose of 200 mg/kg CHF. That study indicated that the genotoxicity distributed to tissues but it was not measured in the host cells. Compared to MX, the order of the genotoxic potency in the test was MX>CMCF>MCA (Fekadu *et al.*, 1994).

### 2.5 Carcinogenicity of MX

The carcinogenicity of MX has been evaluated in one full-range study in rats and in a few shorter studies. MX was carcinogenic, inducing malignant and benign tumors in male and female Wistar rats when it was administered via the drinking water for 104 weeks (Komulainen *et al.*, 1997). Tumors occurred at the average daily doses which were not overtly toxic to rats (0.4, 1.3, 5.0 mg/kg for males and 0.6, 1.9, 6.6 mg/kg for females). MX was a multisite carcinogen. The main target organs of the tumorigenicity were the thyroid glands and the liver.

The thyroid glands were the main target organ in both male and female rats. Fifty-five percent of the high-dose group males and 44% of the high-dose group females suffered follicular carcinoma. The frequencies of follicular adenoma in the high-dose male and female group were 43 and 72%, respectively. Cholangioma, an epithelial tumor of bile duct, was the most prevalent MX-induced liver tumor: 66% of the high-dose group females exhibited cholangioma. The liver tumor incidences were clearly different between males and females. In the high-dose male group, the frequency of cholangioma was only 8%.

In other tissues, MX increased in a statistically significant manner the tumor frequencies in adrenal glands (cortical adenoma in males and females), in pancreas (Langerhans' cell adenoma in males), in mammary glands (adenocarcinoma and fibroadenoma in females) and in the lungs (alveolar and bronchiolar adenomas in males). In female rats, the tumor frequency of lymphomas and leukemias increased in a dose-dependent fashion. In the skin of male rats, the frequency of basal cell tumors increased statistically significantly, but according to the authors, this might

be a chance effect, because no such effect was observed in females or for other skin tumors.

The study has been repeated in Wistar females at the dose of 1.7 mg/kg as a part of the CEMFEC-study (cocarcinogenesis of radiofrequency radiation) with essentially similar tumor spectra and results being obtained (Heikkinen *et al.*, manuscript).

MX increased the number of renal tumors in *Tsc2* (the tuberous sclerosis complex tumor-suppressor gene) mutant Long-Evans (Eker) rats, which have a genetic susceptibility to suffer renal cancer (Hooth *et al.*, 2002). MX was administered to rats in their drinking water at average daily doses of 0.28 or 4.2 mg/kg for males and 0.45 or 7.2 mg/kg for females for 4 or 10 months. After 10 months, statistically significant increases in the number of renal adenomas were observed in the high dose male and female groups compared to control animals.

Some other experiments have been performed to evaluate the carcinogenicity of MX (Nishikawa *et al.*, 1994; 1999), but they were possibly too brief in duration to reveal carcinogenicity. Nishikawa *et al.* (1999) observed that after administration of 57 weeks to MX in drinking water, there was indication of initial signs of carcinogenesis in thyroid glands and bile ducts. The same study demonstrated that MX may promote tumor development.

The carcinogenicity of MX in mice has not been evaluated in a full-range study in conventional animals. MX did not evoke carcinogenicity in the groups of 10 male and female *gpt* delta C57BL/6J transgenic mice, when MX was given via the drinking water at the dose of 100 ppm for 78 weeks (Nishikawa *et al.*, 2005) covering a similar concentration range of MX in drinking water as used in the rat study (between 5.9 – 70.0 ppm, Komulainen *et al.*, 1997). It is uncertain how comparable such a strain is to conventional animals. The group sizes were also small, which may decrease the power to detect carcinogenicity. In mice, no tumors were found in distinct contrast to the situation in rats.

In male and female C57BL/6J-*Min*/+ mice, which are highly susceptible to the spontaneous formation of intestine tumors, MX did not increase statistically significantly the number of tumors in small intestine, colon, or caecum compared to controls (Steffensen *et al.*, 1999). MX was given via the drinking water at average daily doses of 33 and 42 mg/kg for males and females, respectively, for 6 weeks and then the mice received sterile water for 4 additional weeks before the termination of the experiment. The study was short but the strain was anticipated to be sensitive to develop tumors.

The carcinogenicity of CMCF, MCA, and MCF has not been studied in any life-time bioassays.

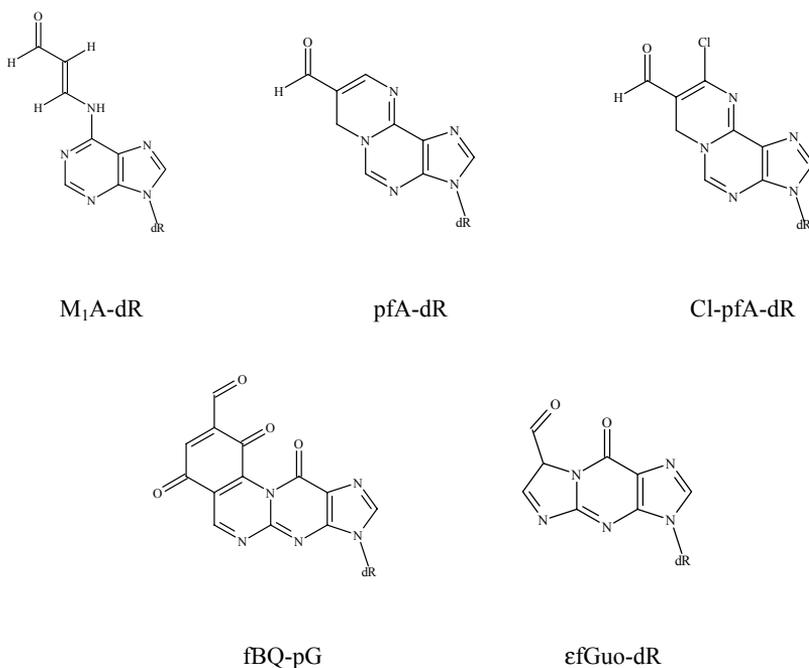
In the short-term intestinal carcinogenesis assay, MCA increased the incidence and the number of aberrant crypt foci in the colons of Balb/cA male mice compared to untreated mice (Steffensen *et al.*, 1999). MCA was given intrarectally at doses of 5 or 10 mg/kg three times per week for four weeks. Prior to the termination of the experiment, mice received regular tap water for twelve additional weeks. Aberrant foci have been considered as an early sign of carcinogenesis. The brief duration of the study and the relatively small number of animals do not allow firm conclusions to be drawn with respect to carcinogenicity.

## 2.6 Mechanisms of action of MX

Though MX has clearly exhibited mutagenic and genotoxic properties in *in vitro* experiments, the primary mechanism of mutagenesis still remains to be clarified. MX has several effects which could contribute to mutagenesis and genotoxicity. It is the combination of these properties which ultimately may account for the mutagenicity of MX.

### 2.6.1 Adduct formation

It is generally accepted that the formation of DNA adducts is one key element in chemical carcinogenesis (Poirier, 2004). DNA adducts are formed *in vitro* in the reactions of MX with adenosine, guanosine, and calf thymus DNA (Le Curieux *et al.*, 1997; Franzén *et al.*, 1998b; Munter *et al.*, 1998; 1999a). The adducts can be characterized by UV absorbance, nuclear magnetic resonance spectroscopy and mass spectrometry. Incubation of 2'-deoxyadenosine with MX at pH 7.4 at 37 °C for eight days resulted in the formation of three different adducts: M<sub>1</sub>A-dR (Le Curieux *et al.*, 1997), pfA-dR, and Cl-pfA-dR (Munter *et al.*, 1998) (Fig. 4). The yields of M<sub>1</sub>A-dR, pfA-dR, and Cl-pfA-dR were 0.01%, 0.03%, and 0.03%, respectively. The adducts M<sub>1</sub>A-dR and pfA-dR were formed also in the reaction of MX with calf thymus DNA at pH 6.5 (M<sub>1</sub>A-dR) or 7.4 (pfA-dR) at 37 °C for 4 days (Le Curieux *et al.*, 1997; Munter *et al.*, 1998). The yields of M<sub>1</sub>A-dR and pfA-dR were 1 and 0.6 adducts/10<sup>5</sup> nucleotides, respectively. The adenine adducts M<sub>1</sub>A-dR and pfA-dR could represent premutagenic lesions. AT→TA transversions have been detected in *S. typhimurium* strain TP2428 induced by MX (Knasmüller *et al.*, 1996).



$M_1A$ -dR: 3-(2'-deoxyribofuranosyl- $N^6$ -adenosinyl)propenal  
 pfA-dR: 3-(2'-deoxy- $\beta$ -D-ribofuranosyl)-7*H*-8-formyl[2,1-*i*]pyrimidopurine  
 Cl-pfA-dR: 3-(2'-deoxy- $\beta$ -D-ribofuranosyl)-7*H*-8-formyl-9-chloro[2,1-*i*]pyrimidopurine  
 $\epsilon$ fGuo-dR: 3-( $\beta$ -D-ribofuranosyl)-7-formylimidazo[1,2-*a*]purin-9(4*H*)-one  
 fBQ-pG: 10-formyl-1, $N^2$ -benzoquinoline propenoguanosine

**Figure 4.** Structures of the MX-DNA adducts.

In the reaction of MX with guanosine, the formation of two adducts has been detected:  $\epsilon$ fGuo (at pH 9, 8.2, 7.4, 6, and 4.6 at 37 °C, Munter *et al.*, 1999a) and fBQ-pG (at pH 7.4 at 37 °C for ten days, Franzén *et al.*, 1998b) (Fig. 4). The yields of the adducts ranged between 0.1 – 0.9% depending on the pH. However, in the repeat study of Munter *et al.* (1999a), the adduct fBQ-pG (Franzén *et al.*, 1998b) could not be detected. Guanine adducts are compatible with the concept that guanine seems to be the main target of MX in DNA (DeMarini *et al.*, 1995; Hyttinen *et al.*, 1996; Knasmüller *et al.*, 1996). However, the  $\epsilon$ fGuo-adduct was not detected in the reaction of MX with calf thymus DNA *in vitro* at a detection limit of five adducts/ $10^7$  nucleotides (Munter *et al.*, 1999a). This may indicate, that DNA lesions at guanine bases occur via a mechanism other than formation of stable DNA

adducts. There is as yet no evidence that any of these five adducts are formed *in vivo*.

Formation of DNA adducts has not been observed in the reactions of MX with thymidine or cytidine (Munter *et al.*, 1999a).

CMCF, MCA, and MCF have also formed adducts. Incubation of calf thymus DNA at physiological pH and temperature for four days with CMCF, MCA, and MCF resulted in the formation of pfA-dR, εcA-dR, and fbaA-dR, respectively (Table 3).

**Table 3.** DNA adducts in the reactions of CMCF, MCA, and MCF with calf thymus DNA.

CHF/adduct/yield/reference
CMCF pfA-dR: 3-(2'-deoxy-β-D-ribofuranosyl)-7H-8-formyl[2,1- <i>i</i> ]pyrimidopurine 6 adducts/10 <sup>5</sup> nucleotides (Munter <i>et al.</i> , 1999b)
MCA εcA-dR: 3-(2'-deoxyribofuranosyl)-7-formylimidazo[2,1- <i>i</i> ]purine 5 adducts/10 <sup>6</sup> nucleotides (Le Curieux <i>et al.</i> , 1997)
MCF fbaA-dR: 4-(2'-deoxyribofuranosyl- <i>N</i> <sup>6</sup> -adenosinyl)-3-formyl-3-butenic acid 4 adducts/10 <sup>5</sup> nucleotides (Le Curieux <i>et al.</i> , 1997)

Incubation of MCA and MCF with nucleosides also resulted in the formation of many adducts (Kronberg *et al.*, 1992; 1993; 1996; Asplund *et al.*, 1995; Munter *et al.*, 1996).

### 2.6.2 Mutations *in vitro*

MX-induced mutations are directed primarily at GC sites in the DNA of bacteria and mammalian cells. In the *hisG46* gene of the *S. typhimurium* strains TA100, TA1535, and TA1950 MX induced mainly GC→TA transversions (DeMarini *et al.*, 1995; Hyttinen *et al.*, 1995; Knasmüller *et al.*, 1996). In strain TA100, MX induced to a lesser extent also GC→AT transitions (DeMarini *et al.*, 1995). Most of these base-pair substitution mutations were in the second position of the *hisG46* (CCC) target codon. In the *hisD3052* gene of strain TA98, MX induced 2-base deletions and complex frameshifts containing primarily GC→TA transversions at the CGCGCGCG site (DeMarini *et al.*, 1995). In the *hisG428* gene of strains TP2428 and TA104, MX induced also mainly GC→TA transversions (target sequence TAA) and to a lesser extent AT→TA transversions (Knasmüller *et al.*, 1996; Shaughnessy *et al.*, 2000). In *E. coli* strains with different DNA repair capabilities MX induced frameshift mutations. The base-pair substitution mutations were mainly GC→TA transversions, but also to some extent GC→AT transitions were detected (Watanabe-Akanuma and Ohta, 1994).

The mutational spectra by MX in mammalian cells is consistent with the mutational spectra in bacteria. In the hypoxanthine phosphoribosyl transferase (*hprt*) gene of CHO cells, most base-pair substitutions were GC→TA transversions. The remaining mutations consisted of AT→TA transversions and deletions of single GC base pairs (-1 frameshifts) (Hyttinen *et al.*, 1996). The mutations involving GC→TA transversions are consistent with the 'A' rule that adenine nucleotides are preferentially added opposite to non-instructional lesions (Strauss, 1991).

The mutational spectras induced by CMCF and MCF in *S. typhimurium* strain TA100 are almost identical with MX. Both chemicals induce primarily GC→TA transversions, in particular, at the second position of the *hisG46* (CCC) target codon. In contrast, MCA evoked predominantly GC→AT transitions in the second position of the CCC codon in the *hisG46* allele (Hyttinen *et al.*, 1995; Knasmüller *et al.*, 1996). In strain TA104, more than half of the mutations induced by MCF were GC→TA transversions, most of the remaining revertants being AT→TA transversions (Shaughnessy *et al.*, 2000).

### 2.6.3 Thermodynamic mechanism

Instead of DNA adduct formation, a thermodynamic mechanism as an alternative mechanism for the mutagenic activity of MX has been suggested (Tuppurainen, 1997). There is, however, no evidence that this mechanism actually occurs in mammalian cells for MX. Studies on the electronic properties of MX have indicated

that the mutagenicity of MX in bacteria is mainly a manifestation of its electron-accepting ability (Tuppurainen *et al.*, 1991; 1992; Tuppurainen, 1992; Tuppurainen and Lötjönen, 1993). The mutagenicity of MX would depend on the electron affinity and the energy of the lowest unoccupied molecular orbital ( $E_{\text{lumo}}$ ). Mutagenicity would increase with decreasing LUMO energy i.e. with increasing electron affinity (Tuppurainen *et al.*, 1991; 1992; LaLonde *et al.*, 1992; Tuppurainen, 1992; Tuppurainen and Lötjönen, 1993). Thus, the mutagenesis would involve an electron transfer from a nucleophilic DNA base to the lowest empty molecular orbital of the MX compound. According to the hard-soft acid-base principle, soft electrophiles (e.g. MX) prefer to bind to soft nucleophiles. Thus, the most favourable interaction of MX would occur with guanine, because it is the softest of the DNA bases (Tuppurainen *et al.*, 1992). The hot spot for the MX-induced mutations in *S. typhimurium* strain TA100 is the second position of the *hisG46* target CCC codon (DeMarini *et al.*, 1995; Hyttinen *et al.*, 1995), which represents the global minimum energy position for a positive hole in ionized DNA. In the thermodynamic mechanism, MX-induced damage at DNA bases would involve a one-electron reduction as a key step leading to an ionized DNA base (e.g.  $\text{CG}^{+\bullet}$ ) (Tuppurainen, 1997). Thus, instead of site-specific binding or adduct formation, the potential mechanism to explain the mutagenic activity of MX would be thermodynamic in its nature.

#### 2.6.4 The promotion effect

There is some evidence *in vivo* that MX may promote tumor development. MX had promoting effects on Wistar rat glandular stomach carcinogenesis after initiation with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and NaCl (Nishikawa *et al.*, 1999). After eight weeks of initiation with MNNG, MX was administered in the drinking water at concentrations of 0, 10, or 30 ppm for 57 weeks. The incidences of adenocarcinomas and atypical hyperplasias in the glandular stomach increased, when MX was given in the drinking water during the postinitiation phase. Furthermore, MX has exhibited enhancing effects on cell proliferation and lipid peroxidation in the glandular stomach mucosa of Wistar rats (Nishikawa *et al.*, 1994) and on cell proliferation in the glandular stomach of F344 rats (Furihata *et al.*, 1992), but a local irritating effect at the site of administration as a contributing factor could not be excluded in the latter study. Wistar and F344 rats were given MX via the drinking water (6.25, 12.5, 25, or 50 ppm for 5 weeks) or by gastric intubation (single dose at 10, 20, 30, or 60 mg/kg), respectively. MX did not lead to formation of tumors in the gastrointestinal tract when given via the drinking water to rats (Komulainen *et al.*, 1997).

MX did not affect the development of the thyroid proliferative lesions in the promotion phase of the rodent two-stage thyroid carcinogenesis model (Son *et al.*, 2000a; 2000b). In the initiation phase, male and female F344 rats were given a single subcutaneous injection of *N*-bis(2-hydroxypropyl)nitrosamine (DHPN) and from one week after the DHPN initiation, MX was administered via the drinking water at a daily dose of 30 ppm for 12 weeks. MX treatment had no influence on thyroid weights, but the relative kidney weights were slightly increased in males (Son *et al.*, 2000b). These observations are compatible with those made in Wistar rats (Komulainen *et al.*, 2000). Thyroid glands are not sensitive to hyperplasia or cell proliferation after brief exposures to MX.

In male Balb/cA mice, MX promoted the growth of aberrant crypt foci in colon but did not increase the number of foci, when the colon carcinogen azoxymethane was given intrarectally for six weeks and then MX (20 or 40 mg/kg) was administered for six or four weeks, respectively (Steffensen *et al.*, 1999). The MX-exposure was rather short for the evaluation of a promoting effect in light of the experience with rats.

The abnormally high tumor incidence in rats, and the finding of Nishikawa *et al.* (1999) on a possible tumor promotion effect for MX, stimulated further studies on MX as a tumor promotion using the two-stage mouse fibroblast cell transformation assay *in vitro*. In those studies, C3H 10T1/2 mouse fibroblasts (Laaksonen *et al.*, 2001) and Balb/c 3T3 mouse fibroblasts (Nakajima *et al.*, 2005) were exposed to 3-methylcholanthrene (MC) in the initiation phase and to MX during the promotion phase of the assay. MX promoted the development of the transformation foci in a dose-dependent manner in both cell lines. In the two-stage C3H 10T1/2 cell transformation assay, MCA and MCF, but not CMCF also enhanced the MC-induced transformation foci formation (Laaksonen *et al.*, 2003). MX, MCA, and MCF promoted the foci formation within the same concentration range (1.0 – 4.0 µg/ml).

In summary, the genotoxicity data on MX suggest that it is a genotoxic carcinogen. The high tumor incidence in certain tissues in rats indicates, however, that other mechanisms may have contributed to tumor development in the experimental studies. The data obtained in mammalian cells suggest that also the other CHF<sub>s</sub>, i.e. CMCF, MCA, and MCF may have properties that contribute to the cancer risk associated with drinking water.

### 3 AIMS OF THE STUDY

The aim of this thesis was to study the cellular mechanisms of MX carcinogenicity in rats to evaluate the cancer risk of MX present in chlorinated drinking water. In the course of the studies, the work was expanded to other CHF<sub>s</sub>, because they proved to be nearly equally potent genotoxins in mammalian cells as MX.

The more specific aims were:

1. To analyze mutation spectras of the *p53* and Ki-, Ha-, and N-*ras* genes in the MX-induced rat liver tumors to elucidate the role of point mutations in those genes in tumor development (**I**).
2. To determine the expression of p53 and p21 Ki-*ras* proteins in the MX-induced rat liver and thyroid gland tumors as an indication of altered activity in the pathways in which these proteins function (**I** and **II**).
3. To define the effects of CHF<sub>s</sub> on GJIC as a possible mechanism of their ability to promote malignant foci formation (**III** and **IV**).

## 4 MATERIALS AND METHODS

### 4.1 Rat tumor samples (I and II)

The liver and the thyroid gland tumor samples for mutation and immunohistochemical analyses were obtained from the 104-week carcinogenicity study with MX in Wistar rats (Komulainen *et al.*, 1997) (Table 4). Only tumors which were large enough to provide a sample were selected for further analysis. The samples represented all dose groups and both sexes but not every single tumor and animal presented in Table 4. The tissue samples had been preserved in 10% buffered formalin at the time of necropsy and embedded in paraffin blocks during the course of the regular histopathology.

### 4.2 Mutation analyses (I)

A total of 50 rat liver tumor samples (16 hepatocellular adenomas, 8 hepatocellular carcinomas, 23 cholangiomas, 3 cholangiocarcinomas) were examined in the mutation analyses of *p53* and *ras* genes. The samples were representative for tumors, but contained also normal tissue. Exons 4-7 of *p53* gene were amplified by polymerase chain reaction (PCR) and mutations in the exons were screened using single-strand conformation polymorphism (SSCP). Direct sequencing was used to verify the SSCP-suggested mutations. Mutation analyses of the PCR amplified exons 1-2 of Ki-, Ha-, and N-*ras* genes were performed using a combination of temperature gradient gel electrophoresis (TGGE), SSCP and DNA sequencing. The exons 4-7 of *p53* and exons 1-2 of *ras* genes were chosen for mutation screening, because mutations in those exons are particularly common in a variety of tumors (Barbacid, 1987; Stanley, 1995; Perantoni and Rice, 1999).

**Table 4.** Rat liver and thyroid tumors in the MX carcinogenicity study (Komulainen *et al.*, 1997).

Sex/tissue/tumor type	Control	MX <sup>a</sup>		
		Low dose	Mid dose	High dose
<b>Females</b>				
Liver				
Adenoma	1 <sup>b</sup> (2%) <sup>c</sup>	1 (2%)	1 (2%)	10 (20%)
Carcinoma	1 (2%)	1 (2%)	3 (6%)	0
Cholangioma	0	4 (8%)	10 (20%)	33 (66%)
Cholangiocarcinoma	1 (2%)	0	0	2 (4%)
Thyroid glands				
Follicular adenoma	4 (8%)	16 (33%)	36 (72%)	36 (72%)
Follicular carcinoma	1 (2%)	3 (6%)	6 (12%)	22 (44%)
C-cell adenoma	11 (22%)	11 (22%)	10 (20%)	16 (32%)
C-cell carcinoma	0	0	0	1 (2%)
<b>Males</b>				
Liver				
Adenoma	0	1 (2%)	2 (4%)	4 (8%)
Carcinoma	0	0	2 (4%)	1 (2%)
Cholangioma	0	0	1 (2%)	4 (8%)
Hepatocholangiocarcinoma	0	1 (2%)	0	0
Thyroid glands				
Follicular adenoma	2 (4%)	20 (40%)	34 (68%)	21 (43%)
Follicular carcinoma	0	1 (2%)	9 (18%)	27 (55%)
C-cell adenoma	11 (22%)	7 (14%)	10 (20%)	11 (22%)
C-cell carcinoma	0	0	2 (4%)	0

<sup>a</sup>MX doses for females: 0.6 (low dose), 1.9 (mid dose), and 6.6 mg/kg (high dose), and for males: 0.4 (low dose), 1.3 (mid dose), and 5.0 mg/kg (high dose) (Komulainen *et al.*, 1997).

<sup>b</sup>Number of animals with one or more tumors indicated.

<sup>c</sup>Frequency of animals with tumor as percentage of examined animals.

#### 4.2.1 DNA extraction

For DNA extraction, 30- $\mu$ m sections were cut from paraffin-embedded blocks of the liver tumor tissues. DNA was extracted with the phenol-chloroform-isoamylalcohol method. DNA was dissolved in TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and kept at  $-20^{\circ}\text{C}$  until used in PCR.

#### 4.2.2 Polymerase chain reaction (PCR)

Amplification of the exons 4-7 of *p53* and exons 1-2 of *ras* genes by PCR was carried out using the extracted DNA as the template. The procedure is described in detail in the original publication (I).

#### 4.2.3 Temperature gradient gel electrophoresis (TGGE)

The TGGE mutation detection method can separate DNA fragments which differ at a single base pair site. The principle of this screening method is that a DNA fragment migrates into a polyacrylamide gel with a gradient of increasing temperature until it reaches a temperature which induces partial strand unwinding. As a consequence of being partially denatured, the DNA undergoes a decrease in its electrophoretic mobility. Homologous DNA fragments differing by a single base pair in the area of mutation migrate to different depths before undergoing abrupt changes in mobility (Wartell *et al.*, 1998).

TGGE and SSCP methods rely on the technique of PCR to amplify the region of DNA of interest. Thus, TGGE was carried out for the PCR products of *ras* genes using a Biometra TGGE system (Göttingen, Germany). Silver staining was used for visualization of the bands.

#### 4.2.4 Single-strand conformation polymorphism (SSCP)

The principle of SSCP mutation detection method is based on the fact that the secondary structure of single-stranded DNA depends on the DNA sequence. Alterations in the secondary structure due to a single base change in the DNA sequence can modify the electrophoretic mobility of DNA fragment (Balogh *et al.*, 2004).

SSCP was carried out for the PCR products of *p53* gene and *ras* genes using a PhastGel electrophoresis system (Amersham Pharmacia Biotech, Uppsala, Sweden) and a Biometra TGGE system, respectively. Silver staining was used for visualization of the bands.

#### 4.2.5 Direct sequencing

The PCR products of the *p53* and *ras* genes were purified and the direct sequencing was performed as described in the original publication (I).

### 4.3 Immunohistochemical analyses (I and II)

Expression of p53 protein was studied in 56 rat liver tumor samples (14 hepatocellular adenomas, 8 hepatocellular carcinomas, 32 cholangiomas, 2 cholangiocarcinomas) (I) and in 34 rat thyroid gland tumor samples (3 follicular adenomas, 29 follicular carcinomas, 2 C-cell carcinomas) (II) by immunohistochemical staining using the polyclonal antibody CM5 (Novocastra Laboratories, Newcastle-upon-Tyne, UK). Expression of p21 Ki-ras protein was evaluated in thyroid gland tumor sections (13 follicular carcinomas, one C-cell carcinoma) using the monoclonal antibody F234 (Santa Cruz Biotechnology, Santa Cruz, CA) (III). CM5 and F234 are assumed to detect both mutated and wild-type p53 and p21 Ki-ras, respectively.

Five micrometer thick sections were cut from paraffin-embedded tissue blocks. Sections were immunohistochemically stained for p53 or p21 Ki-ras protein using the standard avidin-biotin-peroxidase complex method (ABC Vectastain Elite Kit, Vector Laboratories Inc., Burlingame, CA). The samples were considered p53- or p21 Ki-ras-positive when  $\geq 10\%$  cells in the entire tumor area expressed the protein.

### 4.4 Analyses of gap junctional intercellular communication (GJIC) (III and IV)

#### 4.4.1 Cell cultures

Two cell lines were used for the analyses of GJIC. The experiments were done first using Balb/c 3T3 mouse fibroblast cells (clone A31) (III), because the CHF<sub>s</sub> showed promotion potential in mouse fibroblasts (Laaksonen *et al.*, 2001; 2003). To confirm the results in the target cells for MX-induced tumorigenicity, WB-F344 rat liver epithelial cells were used (IV). The Balb/c 3T3 and WB-F344 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, UK) and Eagle's Minimum Essential Medium (EMEM) (Sigma, St. Louis, MO) with the supplements, respectively, at 37 °C in an atmosphere of 5% CO<sub>2</sub>.

#### 4.4.2 Chlorohydroxyfuranones (CHF<sub>s</sub>)

MX was synthesized by Radian International (Austin, TX). CMCF and MCF were synthesized as described by Franzén and Kronberg (1995). MCA was from Sigma-Aldrich (Steinheim, Germany). The chemical structures of the compounds are shown in Fig. 2.

#### 4.4.3 Scrape-loading dye transfer (SL/DT) assay

The effects of CHF<sub>s</sub> on GJIC were studied using the SL/DT assay (El-Fouly *et al.*, 1987). The cells were grown to near confluence in 60-mm culture dishes and the medium was replaced with fresh medium containing the chemical at the desired concentration. The concentrations and exposure times of CHF<sub>s</sub> and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (positive control) used in the assay are summarized in Table 5. The viability of the cell cultures was determined using the Trypan blue dye exclusion (TBDE) test. After the exposure, a Lucifer yellow dye solution (Sigma) was added and the cells were scraped using a razor blade. After dye incubation, the cells were fixed with formaldehyde. The migration of Lucifer yellow dye from the scrape line was examined with an epifluorescence microscope. GJIC was determined by counting the total number of fluorescence stained cells next to the scrape line (III) or by measuring the distance of the dye transfer from the scrape line (IV).

**Table 5.** Concentrations and exposure times of CHF<sub>s</sub> and TPA (positive control) in the SL/DT assay.

	Concentrations (μM)	Exposure times (h)
<b>Balb/c 3T3 cells (III):</b>		
MX	0.005, 0.05, 5, 50	0.25, 0.5, 1, 2, 3, 6
CMCF	50, 75, 100, 125	0.5, 3, 6
MCA	0.05, 0.5, 5, 50	0.5, 3, 6
MCF	100, 250, 625, 1562.5	0.5, 3, 6
TPA	0.002, 0.008, 0.032, 0.128, 8	0.5
<b>WB-F344 cells (IV):</b>		
MX	1.875, 3.75, 7.5, 15	1, 12
CMCF	6.25, 12.5, 25, 50	1, 12
MCA	10, 20, 40, 80	1, 12
MCF	50, 150, 450, 1350	1, 12
TPA	0.01	1, 12

#### 4.4.4 Enzyme inhibitors (IV)

To investigate the role of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) signaling pathways on the inhibition of GJIC by CHF in WB-F344 cells, the enzyme inhibitors GF109203X (bisindolylmaleimide 1) (Sigma) and PD98059 (Sigma) were used to block the activity of the PKC and MAPK, respectively, before exposure to CHF. GF109203X inhibits the isozymes PKC  $\alpha$ ,  $\beta_1$ ,  $\beta_2$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$  (Gekeler *et al.*, 1996). No specific inhibitor of MAPK was available, but PD98059 is a specific inhibitor of MEK 1, which is the direct upstream activator kinase of the MAPK (Dudley *et al.*, 1995).

#### 4.4.5 Western blot (IV)

Western blot analysis was performed to determine the involvement of phosphorylation of connexin43 (Cx43) protein in the inhibition of GJIC by CHF in WB-F344 cells. The analysis was performed as described by Trosko *et al.* (2000). After CHF exposure, the cells were lysed and protein samples were subjected to sodium dodecylsulphate – polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). Cx43 was detected with polyclonal rabbit anti-connexin 43 (Zymed Laboratories Inc., San Francisco, CA) as the primary antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG (Zymed) as the secondary antibody using the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) (Bio-Rad) colorimetric method.

#### 4.4.6 Reverse transcription-polymerase chain reaction (RT-PCR) (IV)

RT-PCR was performed to analyze Cx43 mRNA levels in WB-F344 cells after CHF exposure. Total RNA was isolated using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma). cDNA synthesis was performed using Omniscript reverse transcriptase (Qiagen, Hilden, Germany) and random hexamers (Roche, Mannheim, Germany). After cDNA synthesis, PCR was carried out using DyNAzyme™ II DNA polymerase (Finnzymes, Espoo, Finland) and Cx43-primers (forward: 5'-GCGTGAGGAAAGTACCAAAC-3'; reverse: 5'-GTGAAGCCGCCCAAAGTTG-3').

### 4.5 Measurement of caspase-3-like protease activity (III)

The effects of MX on apoptosis were detected by the measurement of caspase-3-like protease activity. The Balb/c 3T3 cells were grown to near confluence in 60-mm

culture dishes and the medium was replaced with fresh medium containing MX (0.05, 5, 50, 100, 200, 800, and 2000  $\mu$ M) at the desired concentration. After the exposure (1, 2, or 6 h), the caspase-3-like protease activity was measured as described by Movsesyan *et al.* (2002). The cleavage of the fluorogenic substrate, Ac-DEVD-AMC, was measured fluorometrically with a Perkin-Elmer HTS7000 Plus fluorescence plate reader at an excitation wavelength of 380 nm and an emission wavelength of 465 nm.

#### **4.6 Statistical analyses (III and IV)**

The concentration-dependent inhibition and the recovery of the inhibition of GJIC by CHF<sub>s</sub>, and caspase-3 activity by MX were tested using one-way ANOVA followed by Tukey or Dunnett post hoc test (SPSS for Windows version 10.1 or 12.0.1; SPSS, Inc., Chicago, IL). The effects of PKC inhibitor and MEK 1 inhibitor on the inhibition of GJIC by CHF<sub>s</sub> were analyzed using the nonparametric Mann-Whitney test (SPSS).

## 5 RESULTS

### 5.1 Mutation analyses (I)

#### 5.1.1 *p53* gene

Fifty rat liver tumors of the MX carcinogenicity study were analyzed. Of those, two hepatocellular adenomas and one cholangiocarcinoma had four mutations in the *p53* gene (Table 6). Three of the four mutations were GC→AT transitions and one mutation was a AT→TA transversion. All mutations occurred in tumors from the high dose female group. The mutations exhibited no consistent pattern, scattering to different codons and positions of the codon.

**Table 6.** Mutations in *p53* gene in liver tumors of Wistar rats in the MX carcinogenicity study.

Tumor	Nucleotide substitution	Exon	Codon	Predicted amino acid change
Adenoma	GTG→ATG	5	176	Val→Met
	CGG→CGA	7	265	Arg→Arg
Adenoma	AAT→TAT	7	286	Asn→Tyr
Cholangiocarcinoma	CGC→CAC	5	173	Arg→His

#### 5.1.2 *ras* genes

Screening of the mutations in exons 1 and 2 of the Ki-, Ha-, and N-*ras* revealed no mutations in any of the 50 rat liver tumors.

## **5.2 Immunohistochemical analyses (I and II)**

### **5.2.1 Rat liver tumors (I)**

Of the analyzed tumors, all of the cholangiomas (32) and cholangiocarcinomas (2), but not the hepatocellular adenomas (14) or carcinomas (8) overexpressed p53 protein. Cholangiomas and cholangiocarcinomas originate from the bile duct epithelial cells. The epithelial cells of the hyperplastic bile ducts of old rats overexpressed p53 independently of the MX treatment. Livers of the younger rats (up to 24 weeks) did not express p53 protein. Furthermore, MX treatment of the younger rats (30 mg/kg for 18 weeks or 10 mg/kg for 1 or 3 weeks) did not induce expression of the p53 protein. Thus, overexpression of p53 in the bile ducts appeared to be related to the age of the Wistar rats, not to the exposure to MX.

### **5.2.2 Rat thyroid gland tumors (II)**

In most of the studied rat thyroid gland tumor samples there was no expression of the p53 protein. Six follicular carcinomas (21% of those studied) and one C-cell carcinoma (50%) expressed some p53 staining (1-5% of cells positive), but they were not interpreted as being p53-positive. p21 Ki-ras protein expression was not observed in any of the studied 14 thyroid gland tumors, which included also the samples containing some p53 expression.

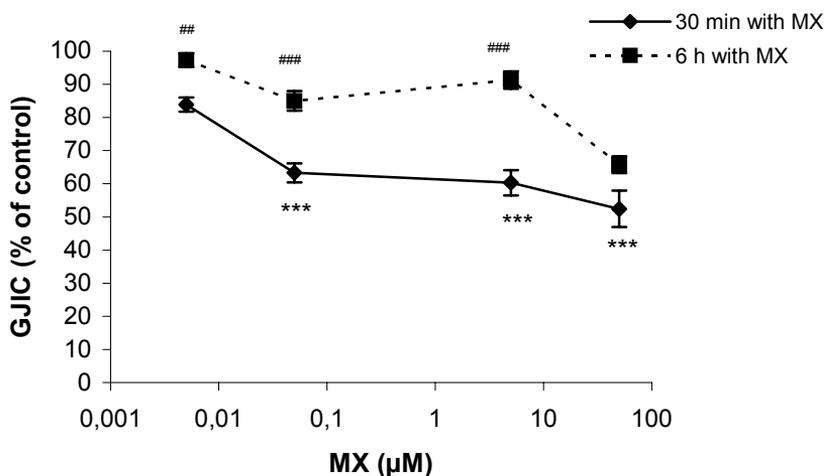
## **5.3 Analyses of gap junctional intercellular communication (GJIC) (III and IV)**

Concentration- and time-responses for inhibition of GJIC by CHF<sub>s</sub> were determined in Balb/c 3T3 (**III**) and WB-F344 cells (**IV**). The involvement of the PKC and MAPK signaling pathways on the inhibition of GJIC by CHF<sub>s</sub> was studied in WB-F344 cells (**IV**). In addition, the effects of CHF<sub>s</sub> on Cx43 protein and mRNA levels were analyzed in WB-F344 cells (**IV**).

In general, GJIC was dose-dependently decreased by all studied CHF<sub>s</sub> in both cell lines. The orders of the potency to inhibit GJIC in Balb/c 3T3 and WB-F344 cells were MX>MCA>CMCF>MCF and MX>CMCF≈MCA>MCF, respectively. However, there were major differences in the potencies and the ranges of the active concentrations of the CHF<sub>s</sub> between the Balb/c 3T3 and WB-F344 cells. Therefore, their effects are summarized compound by compound.

### 5.3.1 Effects of MX on GJIC

In Balb/c 3T3 cells MX inhibited GJIC at nanomolar concentrations. This inhibition occurred at the lowest *in vitro* concentrations observed for any MX-induced effect in mammalian cells so far. The active concentration range was wide. After a 30-min treatment at 5 nM, the inhibition of GJIC was 16% and the maximum inhibition (50%) was achieved at 50  $\mu$ M. After an extended exposure time (up to 6 h), there was some recovery in GJIC (Fig. 5) (III).

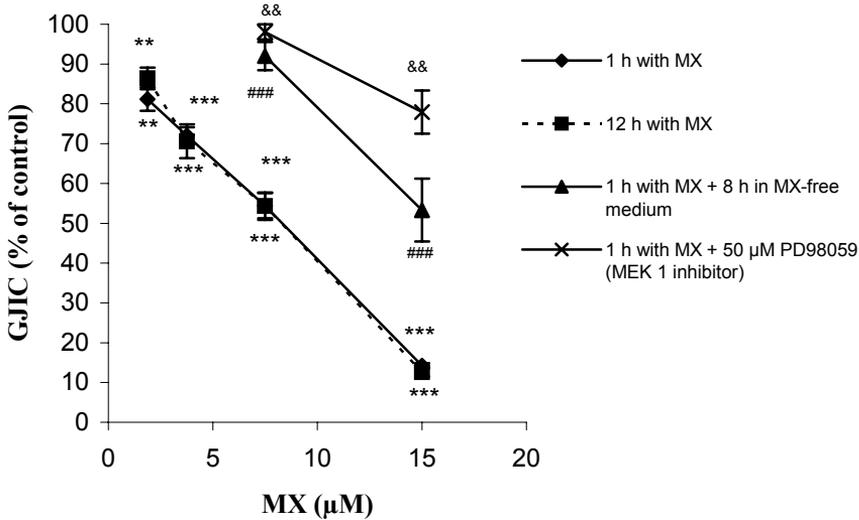


**Figure 5.** Concentration-dependent inhibition of GJIC by MX (mean values  $\pm$  SEM) in Balb/c 3T3 cells. \*\*\* $p$ <0.001, statistically significant difference to negative control (Tukey post-hoc test); ## $p$ <0.01, ### $p$ <0.001, statistically significant difference to 30 min of MX exposure (Dunnett post-hoc test).

In WB-F344 cells, the active concentration range for MX was narrow. After 1 h of exposure at 1.875  $\mu$ M, the inhibition of GJIC was 19%, while the maximum inhibition at 15  $\mu$ M was 85%. The inhibition of GJIC by MX was similar after 12 h of exposure as that seen at 1 h (Fig. 6) (IV).

The MX-induced inhibition of GJIC was a reversible process. When after 1 h of CHF exposure, the WB-F344 cells were incubated in CHF-free medium for up to 8 h, then GJIC recovered slowly (Fig. 6) (IV).

The PKC inhibitor GF109203X had no effect on the inhibition of GJIC by MX in WB-F344 cells, but the MEK 1 inhibitor PD98059 (50  $\mu\text{M}$ , with 5 min pre-exposure) almost completely abolished the inhibitory effect of MX (Fig. 6). Thus, MAPK signaling pathway was involved in the inhibition of GJIC by MX (IV).



**Figure 6.** Concentration-dependent inhibition of GJIC by MX (mean values  $\pm$  SEM), its recovery and effects of MX in combination with the MEK 1 inhibitor on GJIC in WB-F344 cells. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , statistically significant difference to negative control (Dunnett post-hoc test); ### $p < 0.001$ , statistically significant difference to 1 h of MX exposure (Dunnett post-hoc test); && $p < 0.01$ , statistically significant difference to MX exposure alone (Mann-Whitney test).

Three major isoforms of Cx43 protein ranging from unphosphorylated (P0) to increased phosphorylated states (P1, P2) are detectable by Western blot analyses (Matesic *et al.*, 1994). No increases in the basal phosphorylation state of the Cx43 protein were observed in WB-F344 cells after 1 h of exposure to concentrations of MX which inhibited GJIC. In contrast, the levels of all three isoforms of Cx43 were reduced in a concentration-dependent fashion. The Cx43 protein almost completely disappeared after exposure with the highest MX concentration. However, MX caused no appreciable changes in Cx43 mRNA levels in WB-F344 cells.

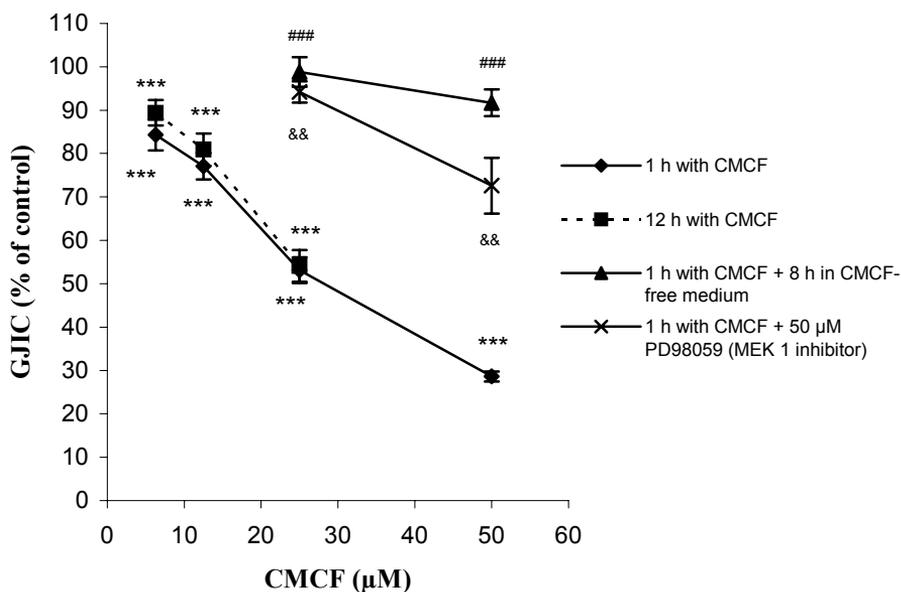
### 5.3.2 Effects of CMCF on GJIC

In terms of the observed lowest effective concentration, CMCF was a 10,000-fold weaker inhibitor of GJIC in Balb/c 3T3 cells than MX. The range of active concentrations was narrow. After 30 min of CMCF treatment, a slight inhibition was seen at 50  $\mu$ M (18%) and the maximum inhibition (50%) was attained at 125  $\mu$ M. During the extended exposure time (up to 6 h), the inhibition of GJIC by CMCF became stronger **(III)**.

In WB-F344 cells, the potency of CMCF to inhibit GJIC was 3.3-fold weaker than that of MX, in terms of the observed lowest effective concentration. The dose-response curve was steep as in Balb/c 3T3 cells. The concentrations needed to inhibit GJIC in WB-F344 cells (Fig. 7) were lower than in Balb/c 3T3 cells. The inhibition was 16% after 1 h of incubation at 6.25  $\mu$ M. Maximal inhibition (70%) occurred at 50  $\mu$ M. As in the case of MX, the inhibition of GJIC by CMCF was continuously down regulated following prolonged exposure (12 h), but the communication was re-established gradually, when CMCF was removed from the system (Fig. 7) **(IV)**.

The inhibitory effect of CMCF was reversed by the MEK 1 inhibitor (Fig. 7), but not by the PKC inhibitor, i.e. activation of MAPK signaling pathway is necessary also for the inhibition of GJIC by CMCF in WB-F344 cells **(IV)**.

The expression of Cx43 protein decreased in a concentration-dependent manner up to total loss at 50  $\mu$ M CMCF, but the mRNA levels did not change **(IV)**.



**Figure 7.** Concentration-dependent inhibition of GJIC by CMCF (mean values  $\pm$  SEM), its recovery, and effects of CMCF in combination with the MEK 1 inhibitor on GJIC in WB-F344 cells. \*\*\* $p < 0.001$ , statistically significant difference to negative control (Dunnett post-hoc test); ### $p < 0.001$ , statistically significant difference to 1 h of CMCF exposure (Dunnett post-hoc test); && $p < 0.01$ , statistically significant difference to CMCF exposure alone (Mann-Whitney test).

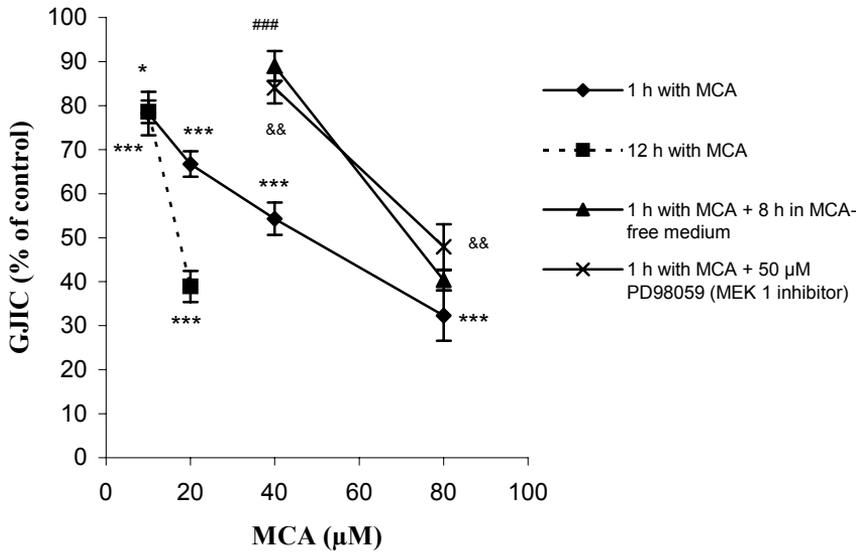
### 5.3.3 Effects of MCA on GJIC

MCA was a 10 times weaker inhibitor of GJIC in Balb/c 3T3 cells than MX. Like MX, MCA exhibited a wide concentration range. A slight inhibition (18%) appeared at 50 nM after 30 min exposure and the maximum inhibition (50%) was achieved at 50  $\mu$ M. There was some recovery in GJIC after the 6 h exposure period (III).

In WB-F344 cells, MCA was a 5.3-fold less potent inhibitor of GJIC than MX, in terms of the observed lowest effective concentration. Unlike in Balb/c 3T3 cells, the range of active concentrations was narrow. After 1 h of exposure, GJIC was inhibited by MCA from 10  $\mu$ M (22%) to a maximum at 80  $\mu$ M (70%). After prolonged treatment (12 h), clearly lower concentrations of MCA inhibited GJIC (Fig. 8). GJIC recovered at 40  $\mu$ M MCA in MCA-free medium, but not at 80  $\mu$ M (Fig. 8), which was already cytotoxic (IV).

The MEK 1 inhibitor counteracted the inhibitory effect of MCA on cell communication (Fig. 8), whereas the PKC inhibitor had no effect. Thus, MAPK signaling pathway also participates in the MCA-induced inhibition of GJIC in WB-F344 cells (IV).

MCA treatment also resulted in a reduction in the expression of Cx43 protein. A complete loss of the expression was observed at 80  $\mu\text{M}$  MCA. However, there were no alterations in the Cx43 mRNA levels (IV).



**Figure 8.** Concentration-dependent inhibition of GJIC by MCA (mean values  $\pm$  SEM), its recovery, and effects of MCA in combination with the MEK 1 inhibitor on GJIC in WB-F344 cells. \* $p < 0.05$ , \*\*\* $p < 0.001$ , statistically significant difference to negative control (Dunnett post-hoc test); ### $p < 0.001$ , statistically significant difference to 1 h of MCA exposure (Dunnett post-hoc test); && $p < 0.01$ , statistically significant difference to MCA exposure alone (Mann-Whitney test).

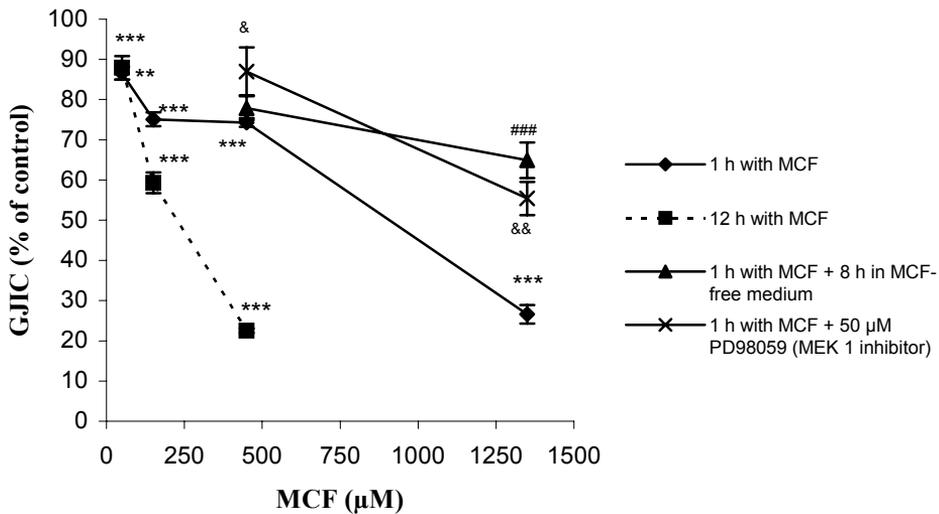
### 5.3.4 Effects of MCF on GJIC

MCF was the weakest inhibitor of GJIC in both cell lines. In Balb/c 3T3 (III) and WB-F344 cells (IV), MCF was 20,000- and 26.7-fold, respectively, weaker inhibitor than MX. The ranges of the active concentrations to inhibit GJIC were similar in both cell lines (100 – 1562.5  $\mu\text{M}$  for Balb/c 3T3 and 50 – 1350  $\mu\text{M}$  for WB-F344). Also in both cell lines, the inhibition of GJIC became stronger after prolonged MCF

treatments. After inhibition by MCF, GJIC reappeared in WB-F344 cells in MCF-free medium (Fig. 9).

The inhibitory effect of MCF was abolished by the MEK 1 inhibitor (Fig. 9), but not by the PKC inhibitor, i.e. the MAPK signaling pathway is involved also on the inhibition of GJIC by MCF in WB-F344 cells (IV).

A concentration-dependent reduction in the levels of all three isoforms of Cx43 protein was observed without any changes in Cx43 mRNA levels also after MCF treatment. A total loss of Cx43 expression was seen at 1350  $\mu$ M MCF (IV).



**Figure 9.** Concentration-dependent inhibition of GJIC by MCF (mean values  $\pm$  SEM), its recovery, and effects of MCF in combination with the MEK 1 inhibitor on GJIC in WB-F344 cells. \*\*p<0.01, \*\*\*p<0.001, statistically significant difference to negative control (Dunnett post-hoc test); ###p<0.001, statistically significant difference to 1 h of MCF exposure (Dunnett post-hoc test); &p<0.05, &&p<0.01, statistically significant difference to MCF exposure alone (Mann-Whitney test).

The main findings of the GJIC analyses are summarized in Table 7.

**Table 7.** The effects of CHF<sub>s</sub> on GJIC, Cx43 protein, and PKC and MAPK signaling pathways.

	<b>MX</b>	<b>CMCF</b>	<b>MCA</b>	<b>MCF</b>
<b>Balb/c 3T3 cells (III)</b>				
The effective concentration range ( $\mu\text{M}$ ) for the inhibition of GJIC (30 min exposure)	0.05 – 50	75 – 125	0.5 – 50	250 – 1562.5
<b>WB-F344 cells (IV)</b>				
The effective concentration range ( $\mu\text{M}$ ) for the inhibition of GJIC (1 h exposure)	1.875 – 15	6.25 – 50	10 – 80	50 – 1350
Recovery of GJIC	GJIC recovered in CHF-free medium.			
The activation of PKC signaling pathway	no	no	no	no
The activation of MAPK signaling pathway	yes	yes	yes	yes
Effect on Cx43 mRNA level	CHF <sub>s</sub> caused no change in the level of Cx43 mRNA.			
Phosphorylation of Cx43 protein	CHF <sub>s</sub> did not increase the basal phosphorylation state of the Cx43 protein.			
Expression of Cx43 protein	CHF <sub>s</sub> caused a concentration-dependent degradation of Cx43 protein.			

### 5.3.5 Cytotoxicity of CHF<sub>s</sub> in GJIC analyses

In Balb/c 3T3 cells, no cytotoxicity was observed at the concentrations of CHF<sub>s</sub> and exposure periods used (TBDE test) **(III)**. In WB-F344 cells, no cytotoxicity was evoked by CHF<sub>s</sub> (TBDE test) after 1 h of exposure at the concentrations used, but incubations for 12 h with 50  $\mu\text{M}$  CMCF, 40  $\mu\text{M}$  MCA, 80  $\mu\text{M}$  MCA, or 1350  $\mu\text{M}$  MCF were cytotoxic to the extent that the GJIC analyses could not be performed **(IV)**.

## **5.4 Apoptosis (III)**

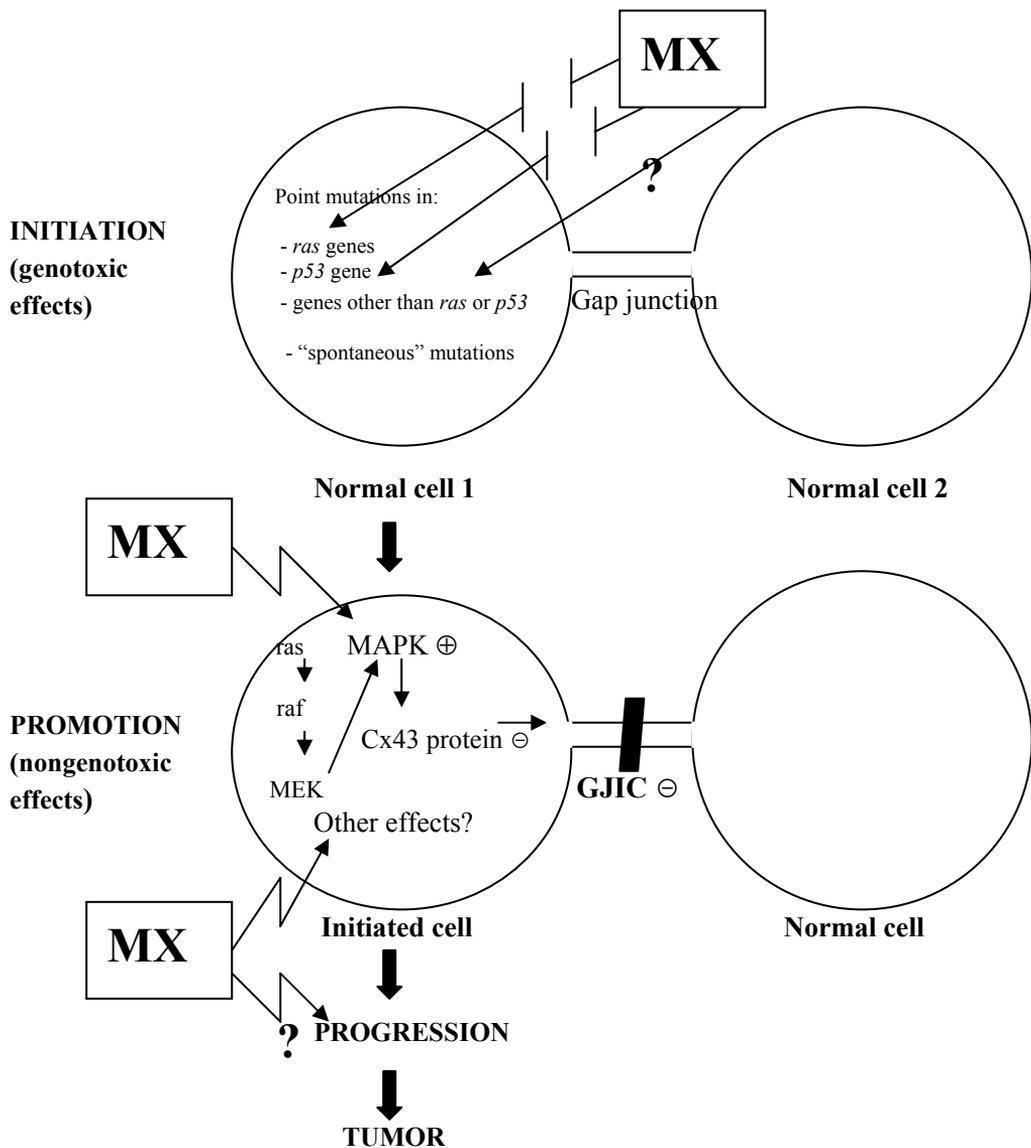
Since GJIC and apoptosis are interrelated, the effects of MX on apoptosis were measured in Balb/c 3T3 cells. MX evoked apoptosis at concentrations 2000 – 4000-fold higher than those needed to inhibit GJIC. There seemed to be a time-dependent concentration window. After 2 h of exposure, an increase in caspase-3 activity appeared only at the 200  $\mu$ M concentration. Almost all cells were undergoing necrosis at 2000  $\mu$ M. After 6 h, the increase in caspase-3 activity occurred also at a slightly lower concentration of 100  $\mu$ M. The majority of the cells were necrotic if exposed to 800  $\mu$ M.

## 6 DISCUSSION

Epidemiological evidence indicates that the consumption of chlorinated drinking water increases the risk of cancer (Cantor *et al.*, 1987; Morris *et al.*, 1992; 1995; Koivusalo *et al.*, 1994; 1995; 1997; Doyle *et al.*, 1997; Villanueva *et al.*, 2004). The detrimental health effects are suspected to be due to some of the several hundreds of different types of organic by-products of chlorination. One candidate group to contribute to the cancer risk in chlorinated drinking water is the CHF's. Of the CHF's, MX is known to be carcinogenic in Wistar rats (Komulainen *et al.*, 1997) but the mechanism(s) to account for the carcinogenicity is/are not known. An improved understanding of the mechanisms of tumor induction would be important in human cancer risk assessment. Therefore, in this thesis, the main goal was to gain insight into the mechanisms behind MX tumorigenicity.

In general, carcinogens can be classified as being either genotoxic (initiating agents) or nongenotoxic (promoting agents). However, many chemicals may act both via genotoxic and nongenotoxic mechanisms, i.e. they are complete carcinogens. Complete carcinogens may have effects at any stage of the carcinogenesis process (Pitot and Dragan, 1991). Besides the fact that many chemical carcinogens operate via a combination of mechanisms, their primary mechanism of action may vary also depending on the target tissues (Barrett, 1993). Thus, according to current knowledge, the multistage model of chemical carcinogenesis (initiation-promotion-progression) (Fig. 1) may have limitations in fully describing the carcinogenesis process.

In order to evaluate the genotoxic potential of MX in rats, mutation and immunohistochemical analyses were performed on the MX-induced liver and thyroid gland tumors, which were the main target organs of the MX tumorigenesis in Wistar rats (Komulainen *et al.*, 1997). Due to observation that MX had potential to promote tumor development *in vitro* (Laaksonen *et al.*, 2001), the nongenotoxic potential of MX was evaluated by performing GJIC studies *in vitro*. The cellular effects of MX in carcinogenesis observed in the current study are presented in Figure 10.



**Figure 10. An illustration of cellular effects of MX in carcinogenesis observed in this study. Initiation phase:** MX did not induce point mutations in *p53* and *ras* genes, though it is a genotoxic compound. The target genes may be genes other than *p53* or *ras* genes and also "spontaneous" mutations may arise. **Promotion phase:** MX promotes the initiated cell by activating an unknown factor(s), which leads to the activation of the MAPK signaling pathway. That, in turn, leads to the decreased expression of Cx43 protein, which results in the inhibition of GJIC. Down-regulation of GJIC could favour the development and growth of the initiated cells into tumor cells.

## 6.1 Mutation analyses (I)

An inherent potential to damage DNA is a characteristic of genotoxic carcinogens. In chemical carcinogenesis, mutations in oncogenes and/or tumor suppressor genes are thought to play a major role in the neoplastic process (Bertram, 2001). In the present study, the mutation analyses were focused on *ras* oncogenes (Ki-, Ha-, and N-*ras*) and the *p53* tumor suppressor gene. *Ras* genes were selected for analyses, because *ras* mutations are often found in chemically-induced rodent tumors (Stanley, 1995; Sills *et al.*, 1999; Takahashi and Wakabayashi, 2004). However, mutation analyses of the MX-induced liver tumors revealed no mutations in *ras* genes. The findings thus suggest that abnormal activation of *ras* genes is unlikely to be involved in the MX-induced rat liver carcinogenesis (Fig. 10).

The *p53* tumor suppressor gene, which is evolutionary highly conserved on the DNA sequence and protein function level and is involved in many cellular pathways, is an important hallmark of the cellular response to genotoxic stress (Pluquet and Hainaut, 2001). Alterations in the *p53* gene are the most common genetic lesions observed in various human cancers (Hainaut and Holstein, 2000). The mutation frequencies of *p53* are lower in rodent tumors compared to human tumors (Stanley, 1995; Miller, 1999). Inactivation of the *p53* gene has been reported in chemically-induced rat liver tumors (Vancutsem *et al.*, 1994; Barbin *et al.*, 1997; Masui *et al.*, 1997; Fu *et al.*, 2003; Deng *et al.*, 2004). The MX-induced liver tumors had only four mutations in the *p53* gene in exons 4-7 (which correspond to human exons 4-8). Three of the four mutations were GC→AT transitions and the fourth was an AT→TA transversion, which is not compatible with the *in vitro* observations. In bacteria and in CHO cells, MX has induced predominantly GC→TA transversions (DeMarini *et al.*, 1995; Hyttinen *et al.*, 1995; 1996; Knasmüller *et al.*, 1996), but these types of mutations were not observed in the liver tumors. Moreover, the mutations in the liver tumors scattered to different codons and positions of the codon. Thus, they exhibited no specific pattern. Mutations in the *p53* gene are found in the progression stage of chemical carcinogenesis (Stanley, 1995; Barbin, 1997; Matsumoto *et al.*, 1997; Miller, 1999). In this study, three mutations occurred in a benign tumor (hepatocellular adenoma) and only one in a malignant tumor (cholangiocarcinoma), however, all of these were found in females of the highest MX dose group. Thus, there was no clear association between mutations and tumor malignancy. These observations together suggest that mutations of the *p53* gene do not play a major role in MX-induced liver carcinogenesis in rats (Fig. 10).

The sensitivity of SSCP to detect mutations is 80 – 85% (Bassett *et al.*, 1998; Yamanoshita *et al.*, 2005). TGGE is more sensitive in detecting mutations, but it is not 100% sensitive (Hass *et al.*, 2000; Frayling, 2002). It is thus possible that all

mutations were not detected but since the detected frequencies were very low, it is most unlikely that possible limitations in the sensitivity of the methods would explain the negative results.

## **6.2 Immunohistochemical analyses (I and II)**

The expression of the p53 protein supports the finding of the mutation analysis. Immunohistochemical analyses were performed to address the role of p53 protein in the MX-induced liver and thyroid tumors and the role of p21 Ki-ras protein in MX-induced thyroid tumors. In general, normal tissue expresses relatively low levels of p53 protein which are often undetectable when examined by immunohistochemical techniques. Instead, mutant p53 proteins (usually missense mutations) are stabilized against intracellular degradation and have prolonged half-lives compared to the short-lived wild-type proteins. Thus, intracellular p53 accumulations, as detectable by immunohistochemical analyses, are believed to be indicative of a DNA damage response and to represent long-lived mutant proteins. However, positively staining tumors are occasionally found that do not carry missense mutations and vice versa, pointing to alternative mechanisms whereby p53 can be stabilized. Thus, immunoreactivity is only an approximate indicator of the tumors with altered p53 function (Harris and Hollstein, 1993).

Several genotoxic carcinogens evoke a rapid overexpression of p53 in rat liver (van Gijssel *et al.*, 1997; Wirtitzer *et al.*, 1998; Nyska *et al.*, 2002). In the current study, no overexpression of p53 protein appeared in the MX-induced hepatocellular adenomas and carcinomas, or in the liver after short-term treatments with MX. Even the hepatocellular adenomas carrying a *p53* mutation, did not overexpress p53 protein. In contrast, all the bile duct tumors, cholangiomas and cholangiocarcinomas, expressed p53 protein, although only one cholangiocarcinoma contained a *p53* mutation. However, the expression of p53 might not be related at all to MX, but rather to the age of rats, because the epithelial cells of the hyperplastic bile ducts of aged rats overexpressed p53 protein independently of MX treatment. As yet unknown factor(s) maintained overexpression of p53 in the bile ducts in aged animals. There is the possibility that those factors can render bile ducts susceptible to MX-induced tumorigenesis.

Mostly chemically-induced thyroid gland tumors in rats are caused by the blood thyroid stimulating hormone (TSH)-mediated hormonal promotion (Thomas and Williams, 1991; McClain, 1995). However, MX did not alter TSH and thyroid hormones thyroxine (T<sub>4</sub>) and triiodothyronine (T<sub>3</sub>) levels in rats after short-term (1 week and 3 weeks) or long-term (104 weeks) administration (Komulainen *et al.*,

1997; 2000). Thus, other mechanisms must be involved in the development of MX-induced tumors in thyroid glands in rats.

Expression of activated wild-type or oncogenic ras proteins can be detected in the premalignant as well as the malignant phase. In malignant tumors, oncogenic ras protein is dominant over wild-type ras (Malumbres and Pellicer, 1998; Singh *et al.*, 2005). Activation of wild-type ras protein can lead to uncontrolled proliferation also in the absence of a mutation. Wild-type ras protein can also antagonize the function of oncogenic ras, but the exact role of wild-type ras protein in growth arrest remains unresolved (Singh *et al.*, 2005). Activation of *ras* oncogenes has been detected both in human and rat thyroid tumors (Lemoine *et al.*, 1988; 1989; Suarez *et al.*, 1990; Kitahori *et al.*, 1995; 1996; 1997; Mizukami *et al.*, 1995; Haugen *et al.*, 1999; Motoi *et al.*, 2000; Kobayashi *et al.*, 2002). A correlation between the immunoreactivity of the ras oncogene product and the extent of *ras* mutations has been detected in the thyroid carcinomas of man (Motoi *et al.*, 2000). It was not possible to perform reliable mutation analyses of the MX-induced thyroid gland tumors, because not enough DNA could be extracted from the specimens on the glass slides. The MX-induced malignant thyroid tumors did not have abnormal expression of p21 Ki-ras protein. Also the response for p53 protein was interpreted as negative. These observations together suggest that the alterations in *p53* and *Ki-ras* gene may not play major roles in the MX-induced thyroid tumorigenesis. The mutation and immunohistochemical analyses indicated that molecular pathways that do not involve inactivation of *p53* or abnormal activation of *ras* genes mediate the MX-induced liver and thyroid gland tumorigenesis in rats.

### **6.3 Gap junctional intercellular communication (GJIC) (III-IV)**

Several nongenotoxic chemicals, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), phenobarbital, saccharin, and peroxisome proliferators, have been proposed to promote the carcinogenic process by acting epigenetically, that is, by causing alterations in the expression of the information of the genome at the transcriptional or posttranscriptional levels (Trosko and Upham, 2005). There is some indication that MX has promoting effects *in vivo* on Wistar rat glandular stomach carcinogenesis (Nishikawa *et al.*, 1999) and MX, MCA, and MCF all promote the development of the transformation foci *in vitro* in the two-stage mouse fibroblast cell (C3H 10T1/2) transformation assay pretreated with MC (Laaksonen *et al.*, 2001; 2003).

One mechanism involved in the tumor-promotion is postulated to be down-regulation of GJIC (Yotti *et al.*, 1979). GJIC has a crucial physiological role in the maintenance of tissue homeostasis (Trosko and Ruch, 1998). Disruption of GJIC

results in loss of homeostatic control and could ultimately be a cause of promotion of tumors (Trosko *et al.*, 1998). The reversible inhibition of GJIC has been demonstrated to be a common phenomenon evoked by many chemical tumor promoters (Trosko and Chang, 1988). Therefore, the effects of CHF on GJIC were investigated. The work was started in mouse Balb/c 3T3 fibroblast cells in which MX was known to promote malignant foci formation.

### 6.3.1 MX

MX enhanced the foci formation both during the initiation and the promotion phase of the two-stage cell transformation assay (Laaksonen *et al.*, 2001). The lowest effective concentration in mouse fibroblast cells (C3H 10T1/2) was 4.6  $\mu$ M MX. Interestingly, the inhibition of GJIC by MX in mouse fibroblast cells (Balb/c 3T3) appeared already at nanomolar concentrations, i.e. at levels clearly below 4.6  $\mu$ M. Actually, the inhibitory effect occurred at the lowest concentrations for MX to cause any effect in mammalian cells *in vitro* so far (Table 8). Thus, the inhibition of GJIC may be one mechanism involved in the promotion of the malignant foci formation by MX. However, the maximum inhibition of GJIC in Balb/c 3T3 cells was only 50%. Balb/c 3T3 cells express Cx43 and Cx32 (Fang *et al.*, 2001). The 50% maximum inhibition could be explained by preferential inhibition of only one type of gap junction, but this is mere speculation and cannot be confirmed from the current results.

Since MX was a potent inhibitor of GJIC in Balb/c 3T3 cells, it raised the intriguing question of whether inhibition of GJIC could be detected also in the target cells of MX tumorigenicity. Thus subsequent GJIC studies were focused on rat liver epithelial cells (WB-F344). MX inhibited GJIC also in WB-F344 cells in a dose-dependent fashion. The observation was confirmed recently by another group (Nishikawa *et al.*, 2006). In the present study, after 1 h of MX exposure, cell coupling recovered slowly in MX-free medium indicating that this was a reversible effect. There was, however, no essential change in the Cx43 mRNA level. Thus, the mechanisms for the alterations of GJIC induced by MX were posttranscriptional.

**Table 8.** The effective concentration ranges of CHF<sub>s</sub> in mammalian cells in GJIC and genotoxicity studies *in vitro*.<sup>a)</sup>

Endpoint	The effective concentration range (μM)			
	MX	CMCF	MCA	MCF
Inhibition of GJIC (Balb/c 3T3 cells) <sup>(III)</sup>	0.05 – 50	75 – 125	0.5 – 50	250 – 1562.5
Inhibition of GJIC (WB-F344 cells) <sup>(IV)</sup>	1.875 – 15	6.25 – 50	10 – 80	50 – 1350
DNA strand breaks	1 – 1000 <sup>(1,2,3,4,5,6)</sup>	27.5 – 880 <sup>(5)</sup>	29.6 – 118.4 <sup>(5)</sup>	1010 – 8080 <sup>(5)</sup>
UDS	2 – 56.25 <sup>(7,8)</sup>	1.5 – 6.25 <sup>(7)</sup>	12.8 – 20 <sup>(7)</sup>	100 <sup>(7)</sup>
Mutations	3.45 – 138 <sup>(9,10,11,12,13,14)</sup>	43.7 – 65.6 <sup>(15)</sup>	23.7 – 47.3 <sup>(16)</sup>	-
SCEs	0.87 – 276 <sup>(1,5,13,17)</sup>	5.5 – 22 <sup>(5)</sup>	8.9 <sup>(5)</sup>	145 – 441 <sup>(5)</sup>
CAs	2.3 – 368 <sup>(5,9,13,17,18)</sup>	22 – 55 <sup>(5)</sup>	11.8 – 14.8 <sup>(5)</sup>	423 – 1616 <sup>(5)</sup>
MN	50 – 100 <sup>(7)</sup>	6.3 – 25 <sup>(7)</sup>	25 <sup>(7)</sup>	100 – 200 <sup>(7)</sup>

<sup>a)</sup>Note: Due to different experimental assays and conditions between the tests, the effective concentrations cannot be exactly compared.

<sup>1</sup>Brunborg *et al.*, 1991; <sup>2</sup>Chang *et al.*, 1991; <sup>3</sup>Holme *et al.*, 1999; <sup>4</sup>Marsteinstredet *et al.*, 1997a; <sup>5</sup>Mäki-Paakkanen *et al.*, 2001; <sup>6</sup>Nunn and Chipman, 1994; <sup>7</sup>Le Curieux *et al.*, 1999; <sup>8</sup>Nunn *et al.*, 1997; <sup>9</sup>Harrington-Brock *et al.*, 1995; <sup>10</sup>Hyttinen *et al.*, 1996; <sup>11</sup>Jansson and Hyttinen, 1994; <sup>12</sup>Matsumura *et al.*, 1994; <sup>13</sup>Mäki-Paakkanen *et al.*, 1994; <sup>14</sup>Woodruff *et al.*, 2001; <sup>15</sup>Niittykoski *et al.*, 1995; <sup>16</sup>Jansson *et al.*, 1995; <sup>17</sup>Jansson *et al.*, 1993; <sup>18</sup>Meier *et al.*, 1987a.

Activation of signal transduction pathways are known to regulate GJIC. The action of a number of kinases may affect a wide variety of Cx processes ranging from the expression of Cx genes to the degradation of the gap junction channels (Cruciani and Mikalsen, 2002; Hervé and Sarrouilhe, 2002). The mechanisms by which TPA, a well known potent tumor promoter and a positive control in the current studies, inhibits GJIC in rat liver epithelial cells, involve hyperphosphorylation of Cx43 and activation of both PKC and MAPK signaling pathways (Lampe *et al.*, 2000; Rivedal and Opsahl, 2001; Ruch *et al.*, 2001; Rivedal and Leithe, 2005). Therefore, the role of hyperphosphorylation of Cx43 and those of PKC and MAPK signaling pathways on the inhibition of GJIC by CHF<sub>s</sub> were evaluated in WB-F344 cells.

Of the post-translational modifications, hyperphosphorylation of Cx is thought to represent a major mechanism of aberrant GJIC induction by chemical agents (Yamasaki and Naus, 1996). However, sometimes the phosphorylation status of Cx can remain unchanged even though GJIC is inhibited (Guan *et al.*, 1995; Sai *et al.*, 1998; Suzuki *et al.*, 2000). WB-F344 cells contain mainly Cx43 protein (Ruch *et al.*, 1994). Treatment of the WB-F344 cells with MX did not increase the basal level of phosphorylation of Cx43. Thus, the mechanisms responsible for aberrant GJIC induced by MX do not seem to involve hyperphosphorylation of Cx43. Instead, it is likely that MX affected GJIC by altering the Cx43 expression. The expression of all of the isoforms of Cx43 protein were reduced. Complete degradation of the Cx43 protein was observed at the highest MX concentration. Reduction of the available Cx43 protein gradually leads to fewer and fewer gap junctions and to inhibition of GJIC.

With prolonged TPA treatment, GJIC was re-established. Proteosomal degradation of PKC is one mechanism to account for this restoration (Leithe *et al.*, 2003). The mechanisms for the inhibition of GJIC by MX appear to be different from those of TPA. The inhibitory response of MX was independent of the exposure period and inhibition of PKC, which means that MX did not inhibit GJIC by activating PKC. Instead, the inhibition of GJIC by MX was clearly dependent upon the activation of the MAPK signaling pathway. It has been demonstrated that tumors exhibiting increased activity of MAPK pathways may progress towards more advanced tumor states in the absence of Cx32-mediated GJIC (King and Lampe, 2004). The MAPKs, which belong to the ras/raf/MEK/MAPK signaling pathway (Fig.10), regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis (Rodriguez-Viciano *et al.*, 2004). Activation of the ras/raf/MEK/MAPK pathway seems to make a pivotal contribution in driving growth and progression of tumors in humans (Thompson and Lyons, 2005). The members of the *ras* gene family encode for 21-kDa proteins (p21ras) that are localized on the inner surface of the plasma membrane and have GTP/GDP binding capacity (Barbacid, 1987). Ras is active in the GTP-bound form and inactive in the GDP-bound form. Mutation of a *ras* gene converts that gene into the active oncogene, which results in the ras protein remaining predominantly bound to GTP compared with the wild-type variants. Activated ras stimulates raf activity, which then switches on MEK and this in turn activates MAPK (Rodriguez-Viciano *et al.*, 2004). However, activation of MAPK does not invariably occur via mutations of *ras* genes (Pesse *et al.*, 2005; Zuidervaart *et al.*, 2005).

ROS have been observed to modulate GJIC (Hu and Cotgreave, 1995; Kojima *et al.*, 1996; Upham *et al.*, 1997). MAPKs are also known to be activated by oxidative stress (Stevenson *et al.*, 1994). MX induced ROS production in murine L929

fibrosarcoma cells but only at relatively high concentrations (100 – 1000  $\mu\text{M}$ ) (Zeni *et al.*, 2004). If high MX concentrations are needed for ROS production in all cells, then ROS cannot probably contribute to the MX-induced carcinogenesis.

The exact site(s) of action of MX remains to be elucidated but these present mechanistic studies suggest that nongenotoxic effects may be contributing to the carcinogenicity of MX (Fig. 10).

### 6.3.2 CMCF

CMCF was classified as an initiator, but not as a promoter in the two-stage mouse fibroblast cell transformation assay (Laaksonen *et al.*, 2003). It even decreased the number of foci induced by MC. Compared to MX, CMCF was a 10,000-fold weaker inhibitor of GJIC in Balb/c 3T3 cells and higher concentrations were needed to evoke the inhibitory effect on cell communication than to affect the foci formation. The low potential of CMCF to inhibit GJIC is compatible with its lack of potency to promote foci formation in those cells. However, in WB-F344 cells, CMCF had similar effects on gap junctions as MX and it was only a 3.3-fold weaker inhibitor of GJIC than MX. Moreover, the concentrations needed to inhibit GJIC in WB-F344 cells were lower than those required in Balb/c 3T3 cells. This may mean that for CMCF the GJIC-inhibiting effect is tissue- or species-specific. CMCF is definitively a genotoxic agent *in vitro*, but the responses related to tumor promotion leave its role in tumor promotion as an open question. CMCF has evoked the genotoxic effects in mammalian cells *in vitro* at the similar concentration ranges as it inhibits GJIC (Table 8).

### 6.3.3 MCA

MCA was a promoter, but not an initiator, in the two-stage mouse fibroblast cell transformation assay (the lowest effective concentration: 5.9  $\mu\text{M}$ ) (Laaksonen *et al.*, 2003). MCA inhibited GJIC in Balb/c 3T3 cells at concentrations below those needed for promotion of the foci formation. Thus, the inhibition of the cell-cell communication might facilitate the foci formation in the mouse fibroblasts. The finding that MCA blocked GJIC also in WB-F344 cells, apparently by the same mechanisms as MX, support the concept that MCA could act as a tumor promoter. MCA inhibited GJIC at the lower or similar concentrations than those needed to cause genotoxicity in mammalian cells *in vitro* (Table 8).

#### 6.3.4 MCF

MCF acted as both an initiator and a promoter in the two-stage mouse fibroblast cell transformation assay (Laaksonen *et al.*, 2003). MCF inhibited GJIC also in Balb/c 3T3 cells. The concentrations needed to inhibit GJIC were clearly higher than those known to be effective in the promotion phase of the transformation assay (the lowest effective concentration: 6.7  $\mu\text{M}$ ). MCF blocked GJIC also in WB-F344 cells at a similar concentration range as in Balb/c 3T3 cells. The concentrations of MCF needed to inhibit GJIC were similar as those required to evoke the genotoxic effects in mammalian cells *in vitro* (Table 8). Overall, MCF was the weakest inhibitor of GJIC in both cell lines and it possessed only a low potential to inhibit GJIC. Thus, other mechanisms seem to be involved in the foci formation caused by MCF.

Taken together, the current results show that MX inhibits GJIC by altering the expression of Cx43 in the cells of one target tissue of its carcinogenicity. Cxs are a multigene family. Each cell type has its own characteristic pattern of Cx proteins. Cx32 is the major Cx expressed by rat hepatocytes. Instead, cholangiocytes (bile duct epithelial cells) express Cx43 as their predominant Cx protein (Neveu *et al.*, 1995; Bode *et al.*, 2002). Normal rat thyroid follicular cells express Cx43, Cx32, and Cx26 (Meda *et al.*, 1993). MX induced especially cholangiomas, epithelial tumors of the bile duct, and thyroid follicular tumors in rats (Komulainen *et al.*, 1997). At present it is not known whether one type of connexins is more sensitive to MX than others and could that explain the carcinogenicity of MX in general or its tissue specificity. A specifically altered expression of Cx43 could well explain MX's propensity to evoke cholangiomas.

### 6.4 Effects of MX on apoptosis (III)

Many tumor promoters inhibit both GJIC and apoptosis. A functional GJIC system may be necessary for initiation of apoptosis and thus inhibition of GJIC would inhibit apoptosis and in that way support tumor promotion (Trosko and Goodman, 1994). Apoptosis has been observed also in the absence of functional gap junctions (Kolaja *et al.*, 2000; Mally and Chipman, 2002) i.e. they are necessarily not always interrelated. Gap junctions may serve a regulatory function between cell growth and apoptosis (Wilson *et al.*, 2000).

MX caused apoptosis in Balb/c 3T3 cells but at much higher concentrations and after longer incubations than those needed to inhibit GJIC. Apoptosis occurred only in a narrow concentration window of 100 – 200  $\mu\text{M}$ . In promyelocytic leukemic HL-60 cells, a similar window-effect (30 – 100  $\mu\text{M}$ ) for MX-induced apoptosis has been observed (Marsteinstredet *et al.*, 1997b). The authors suggested that the DNA in

cells exposed to 300  $\mu\text{M}$  MX was “arrested” at an early apoptotic stage with altered chromatin. It would be interesting to determine whether MX inhibits apoptosis at concentrations which do not yet induce apoptosis. Inhibition rather than induction would support tumor growth and development.

## 6.5 Implications for risk assessment

Risk assessment of chlorinated drinking water is complicated because of the huge numbers and different types of DBPs. There may be also marked interactions between DBPs, which should be taken into account in risk assessment. The observation that a cancer risk is associated with bacterial mutagenicity of drinking water suggests that mutagenic/genotoxic compounds may increase the risk. CHF<sub>s</sub> represent a group whose genotoxicity in mammalian cells has been definitively observed *in vitro*.

MX was evaluated as possibly carcinogenic to humans by IARC (IARC, 2004). An important question is whether MX is a complete carcinogen, a tumor initiator and promoter. This consideration would make it a more potent carcinogen than initially thought. MX is clearly a genotoxic compound in mammalian cells *in vitro* (Table 1), but there is some inconsistency in its genotoxic potency *in vivo* (Tables 2a and 2b). Both the two-stage cell transformation assay and GJIC studies suggest that MX is also a tumor promoter, but this effect *in vivo* has not been evaluated. The classical multistage model of chemical carcinogenesis holds the view that the initial event is a genotoxic one, where the chemical carcinogen induces mutations in oncogenes and/or tumor suppressor genes and these initiated cells are then promoted by nongenotoxic mechanisms turning them into a malignant genotype (Pitot, 1993). An alternate model of chemical carcinogenesis suggests that most chemical carcinogens work through nongenotoxic mechanisms. In that model, mutations in oncogenes are thought to be caused by some independent “spontaneous” event and these spontaneously-initiated cells, induced by either errors in repair of endogenous or exogenous agents or by errors in replication of normal DNA templates, are then promoted by the nongenotoxic action of the chemical, i.e. the chemical is an epigenetic agent (Trosko, 1997; Trosko, 2001; Trosko and Upham, 2005). In both models, MX could be a carcinogen, but tumor promoters are more likely have threshold level of action, i.e. they need higher concentrations for their action compared to genotoxic compounds. Exposure to tumor promoters must also be regular and sustained (which is the case for drinking water by-products), otherwise the process of promotion can be interrupted (Trosko and Upham, 2005).

The carcinogenicity of CHF<sub>s</sub> other than MX is still unexplored, but these compounds are genotoxic in mammalian cells. The present studies revealed that in

rat liver epithelial cells, the potencies of CMCF and MCA to inhibit GJIC were not much lower than the potency of MX. Thus, the other CHF's cannot be excluded from any risk assessment. However, more information about their *in vivo* effects are needed before their contribution to the risks associated with drinking chlorinated water can be assessed.

## 7 CONCLUSIONS

1. In the MX-induced rat liver tumors, no mutations in *Ki-*, *Ha-*, and *N-ras* oncogenes and only four mutations in the *p53* tumor suppressor gene were detected. These results point to the presence of alternative molecular pathways that do not involve point mutations in *ras* genes and *p53* gene.
2. MX-induced rat hepatocellular adenomas and carcinomas did not express p53 protein, which is in good agreement with the mutation data. Instead, all the cholangiomas and cholangiocarcinomas overexpressed p53 protein, but this was proposed to be associated with the age of the rats. p53 and p21 *Ki-ras* proteins were not expressed abnormally in rat thyroid tumors. Thus, these immunohistochemical findings support the concept that mutations in *Ki-ras* and *p53* genes are not targets in the MX-induced tumorigenesis.
3. CHF<sub>s</sub> inhibited GJIC in Balb/c 3T3 mouse fibroblast cells and in WB-F344 rat liver epithelial cells, the target cells of MX tumorigenicity. MX was the most potent inhibitor of GJIC in both cell lines. Considering the effective concentrations of each CHF in Balb/c 3T3 cells, the inhibition of GJIC may be one mechanism through which MX and MCA are involved in the promotion of malignant foci formation in mouse fibroblasts. In WB-F344 cells, CHF<sub>s</sub> inhibited GJIC through the activation of MAPK signaling pathway and by altering Cx43 expression. The inhibition of GJIC by MX may have relevance as a nongenotoxic mechanism involved in MX-induced carcinogenesis.

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## 9 REFERENCES

- Andrews, R.C., Daignault, S.A., Laverdure, C., Williams, D.T., Huck, P.M., 1990. Occurrence of the mutagenic compound 'MX' in drinking water and its removal by activated carbon. *Environ. Technol.* 11, 685-694.
- Asplund, D., Kronberg, L., Sjöholm, R., Munter, T., 1995. Reaction of mucochloric acid with adenosine: Formation of 8-(*N*<sup>6</sup>-adenosinyl)ethenoadenosine derivatives. *Chem. Res. Toxicol.* 8, 841-846.
- Backlund, P., Wondergem, E., Voogd, K., de Jong, A., 1989a. Influence of chlorination pH and chlorine dose on the formation of mutagen activity and the strong bacterial mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-5(2*H*)-furanone (MX) in water. *Chemosphere* 18, 1903-1911.
- Backlund, P., Wondergem, E., Voogd, K., de Jong, A., 1989b. Mutagenic activity and presence of the strong mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2-(5*H*)-furanone (MX) in chlorinated raw and drinking waters in the Netherlands. *Sci. Total Environ* 84, 273-282.
- Balogh, K., Patócs, A., Majnik, J., Rácz, K., Hunyady, L., 2004. Genetic screening methods for the detection of mutations responsible for multiple endocrine neoplasia type 1. *Mol. Genet. Metab.* 83, 74-81.
- Barbacid, M., 1987. *Ras* genes. *Annu. Rev. Biochem.* 56, 779-827.
- Barbin, A., Froment, O., Boivin, S., Marion, M.J., Belpoggi, F., Maltoni, C., Montesano, R., 1997. p53 gene mutation pattern in rat liver tumors induced by vinyl chloride. *Cancer Res.* 57, 1695-1698.
- Barletta, E., Gorini, G., Vineis, P., Miligi, L., Davico, L., Mugnai, G., Ciolli, S., Leoni, F., Bertini, M., Matullo, G., Costantini, A.S., 2004. *Ras* gene mutations in patients with acute myeloid leukaemia and exposure to chemical agents. *Carcinogenesis* 25, 749-755.
- Barrett, J.C., 1993. Mechanisms of multistep carcinogenesis and carcinogen risk assessment. *Environ. Health Perspect.* 100, 9-20.
- Bassett, J.H., Forbes, S.A., Pannett, A.A., Lloyd, S.E., Christie, P.T., Wooding, C., Harding, B., Besser, G.M., Edwards, C.R., Monson, J.P., Sampson, J., Wass, J.A., Wheeler, M.H., Thakker, R.V., 1998. Characterization of mutations in patients with multiple endocrine neoplasia type 1. *Am. J. Hum. Genet.* 62, 232-244.
- Bertram, J.S., 2001. The molecular biology of cancer. *Mol. Aspects Med.* 21, 167-223.
- Bode, H.-P., Wang, L., Cassio, D., Leite, M.F., St-Pierre, M.V., Hirata, K., Okazaki, K., Sears, M.L., Meda, P., Nathanson, M.H., Dufour, J.-F., 2002. Expression and regulation of gap junctions in rat cholangiocytes. *Hepatology* 36, 631-640.
- Bos, J.L., 1989. *ras* oncogenes in human cancer: a review. *Cancer Res.* 49, 4682-4689.
- Brunborg, G., Holme, J.A., Söderlund, E.J., Hongslo, J.K., Vartiainen, T., Lötjönen, S., Becher, G., 1991. Genotoxic effects of the drinking water mutagen 3-chloro-4-

- (dichloromethyl)-5-hydroxy-2[5H]-furanone (MX) in mammalian cells in vitro and in rats in vivo. *Mutat. Res.* 260, 55-64.
- Cantor, K.P., Hoover, R., Hartge, P., Mason, T.J., Silverman, D.T., Altman, R., Austin, D.F., Child, M.A., Key, C.R., Marrett, L.D., Myers, M.H., Narayana, A.S., Levin, L.I., Sullivan, J.W., Swanson, G.M., Thomas, D.B., West, D.W., 1987. Bladder cancer, drinking water source, and tap water consumption: A case-control study. *J. Natl. Cancer Inst.* 79, 1269-1279.
- Chang, L.W., Daniel, F.B., DeAngelo, A.B., 1991. DNA strand breaks induced in cultured human and rodent cells by chlorohydroxyfuranones – mutagens isolated from drinking water. *Teratog. Carcinog. Mutagen.* 11, 103-114.
- Chipman, J.K., Mally, A., Edwards, G.O., 2003. Disruption of gap junctions in toxicity and carcinogenicity. *Toxicol. Sci.* 71, 146-153.
- Cozzie, D.A., Kanniganti, R., Charles, M.J., Johnson, J.D., Ball, L.M., 1993. Formation and characterization of bacterial mutagens from reaction of the alternative disinfectant monochloramine with model aqueous solutions of fulvic acid. *Environ. Mol. Mutagen.* 21, 237-246.
- Cruciani, V., Mikalsen, S.-O., 2002. Connexins, gap junctional intercellular communication and kinases. *Biol. Cell.* 94, 433-443.
- Daniel, F.B., Olson, G.R., Stober, J.A., 1991. Induction of gastrointestinal tract nuclear anomalies in B6C3F1 mice by 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone and 3,4-(dichloro)-5-hydroxy-2[5H]-furanone, mutagenic byproducts of chlorine disinfection. *Environ. Mol. Mutagen.* 17, 32-39.
- DeMarini, D.M., Abu-Shakra, A., Felton, C.F., Patterson, K.S., Shelton, M.L., 1995. Mutational spectra in *Salmonella* of chlorinated, chloraminated, or ozonated drinking water extracts: Comparison to MX. *Environ. Mol. Mutagen.* 26, 270-285.
- Deng, W.-G., Fu, Y., Li, Y.-L., Sugiyama, T., 2004. Potential role of p53 mutation in chemical hepatocarcinogenesis of rats. *World J. Gastroenterol.* 10, 46-52.
- Diamandis, E.P., 1997. Clinical applications of tumor suppressor genes and oncogenes in cancer. *Clin. Chim. Acta* 257, 157-180.
- Doyle, T.J., Zheng, W., Cerhan, J.R., Hong, C.-P., Sellers, T.A., Kushi, L.H., Folsom, A.R., 1997. The association of drinking water source and chlorination by-products with cancer incidence among postmenopausal women in Iowa: A prospective cohort study. *Am. J. Public Health* 87, 1168-1176.
- Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J., Saltiel, A.R., 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA* 92, 7686-7689.
- Egorov, A.I., Tereschenko, A.A., Altshul, L.M., Vartiainen, T., Samsonov, D., LaBrecque, B., Mäki-Paakkanen, J., Drizhd, N.L., Ford, T.E., 2003. Exposures to drinking water chlorination by-products in a Russian city. *Int. J. Hyg. Environ. Health* 206, 539-551.
- El-Fouly, M.H., Trosko, J.E., Chang, C.C., 1987. Scrape-loading and dye transfer. A rapid and simple technique to study gap junctional intercellular communication. *Exp. Cell Res.* 168, 422-430.

- Fang, M.-Z., Mar, W.-C., Cho, M.-H., 2001. Cadmium-induced alterations of connexin expression in the promotion stage of in vitro two-stage transformation. *Toxicology* 161, 117-127.
- Fawell, J.K., Horth, H., 1990. Assessment and identification of genotoxic compounds in water. In: Waters, M.D. (ed.), *Genetic Toxicology of Complex Mixtures*, Plenum Press, New York, pp. 197-214.
- Fekadu, K., Parzefall, W., Kronberg, L., Franzen, R., Schulte-Hermann, R., Knasmüller, S., 1994. Induction of genotoxic effects by chlorohydroxyfuranones, byproducts of water disinfection, in *E. coli* K-12 cells recovered from various organs of mice. *Environ. Mol. Mutagen.* 24, 317-324.
- Franzén, R., Goto, S., Tanabe, K., Morita, M., 1998a. Genotoxic activity of chlorinated butenoic acid in *Salmonella typhimurium* strains TA98, TA100 and TA104. *Mutat. Res.* 417, 31-37.
- Franzén, R., Kronberg, L., 1995. Synthesis of chlorinated 5-hydroxy-4-methyl-2(5H)-furanones and mucochloric acid. *Tetrahedron Lett.* 36, 3905-3908.
- Franzén, R., Tanabe, K., Morita, M., 1998b. Isolation of a MX-guanosine adduct formed at physiological conditions. *Chemosphere* 36, 2803-2808.
- Frayling, I.M., 2002. Methods of molecular analysis: mutation detection in solid tumours. *Mol. Pathol.* 55, 73-79.
- Fu, Y., Deng, W., Kawarada, Y., Kawagoe, M., Ma, Y.-Z., Li, X., Guo, N., Kameda, T., Terada, K., Sugiyama, T., 2003. Mutation and expression of the *p53* gene during chemical hepatocarcinogenesis in F344 rats. *Biochim. Biophys. Acta* 1628, 40-49.
- Furihata, C., Yamashita, M., Kinae, N., Matsushima, T., 1992. Genotoxicity and cell proliferative activity of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone [MX] in rat glandular stomach. *Wat. Sci. Tech.* 25, 341-345.
- Gekeler, V., Boer, R., Uberall, F., Ise, W., Schubert, C., Utz, I., Hofmann, J., Sanders, K.H., Schachtele, C., Klemm, K., Grunicke, H., 1996. Effects of the selective bisindolylmaleimide protein kinase C inhibitor GF 109203X on P-glycoprotein-mediated multidrug resistance. *Br. J. Cancer* 74, 897-905.
- Guan, X., Bonney, W.J., Ruch, R.J., 1995. Changes in gap junction permeability, gap junction number, and connexin43 expression in lindane-treated rat liver epithelial cells. *Toxicol. Appl. Pharmacol.* 130, 79-86.
- Guimaraes, D.P., Hainaut, P., 2002. TP53: a key gene in human cancer. *Biochimie* 84, 83-93.
- Haataja, L., Vartiainen, T., Lampelo, S., Lötjönen, S., Tuomisto, J., 1991. Binding of the strong bacterial mutagen, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) to bovine serum albumin. *Toxicol. Lett.* 59, 187-195.
- Hainaut, P., Hollstein, M., 2000. *p53* and human cancer: the first ten thousand mutations. *Adv. Cancer Res.* 77, 81-137.
- Harrington-Brock, K., Doerr, C.L., Moore, M.M., 1995. Mutagenicity and clastogenicity of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) in L5178Y/TK<sup>+/+</sup>-3.7.2C mouse lymphoma cells. *Mutat. Res.* 348, 105-110.

- Harris, C.C., Hollstein, M., 1993. Clinical implications of the p53 tumor-suppressor gene. *N. Engl. J. Med.* 329, 1318-1327.
- Hass, J., Mayer, K., Rott, H.-D., 2000. Tuberous sclerosis type 1: three novel mutations detected in exon 15 by a combination of HDA and TGGE. *Hum. Mutat.* 16, 88.
- Haugen, D.R.F., Lillehaug, J.R., Aksten, L.A., 1999. Enhanced expression of EGF receptor and low frequency of *ras* mutations in X-ray-induced rat thyroid tumours. *Virchows Arch.* 435, 434-441.
- Hemming, J., Holmbom, B., Reunanen, M., Kronberg, L., 1986. Determination of the strong mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone in chlorinated drinking and humic waters. *Chemosphere* 15, 549-556.
- Hervé, J.C., Sarrouihe, D., 2002. Modulation of junctional communication by phosphorylation: protein phosphatases, the missing link in the chain. *Biol. Cell.* 94, 423-432.
- Holmbom, B., Kronberg, L., Smeds, A., 1989. Chemical stability of the mutagens 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) and E-2-chloro-3-(dichloromethyl)-4-oxo-butenic acid (E-MX). *Chemosphere* 18, 2237-2245.
- Holmbom, B., Voss, R.H., Mortimer, R.D., Wong, A., 1984. Fractionation, isolation, and characterization of Ames mutagenic compounds in kraft chlorination effluents. *Environ. Sci. Technol.* 18, 333-337.
- Holme, J.A., Haddeland, U., Haug, K., Brunborg, G., 1999. DNA damage induced by the drinking water mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5*H*]-furanone (MX) in mammalian cells in vitro and in mice. *Mutat. Res.* 441, 145-153.
- Hooth, M.J., McDorman, K.S., Hester, S.D., George, M.H., Brooks, L.R., Swank, A.E., Wolf, D.C., 2002. The carcinogenic response of *Tsc2* mutant Long-Evans (Eker) rats to a mixture of drinking water disinfection by-products was less than additive. *Toxicol. Sci.* 69, 322-331.
- Hu, J., Cotgreave, I.A., 1995. Glutathione depletion potentiates 12-*O*-tetradecanoyl phorbol-13-acetate(TPA)-induced inhibition of gap junctional intercellular communication in WB-F344 rat liver epithelial cells: relationship to intracellular oxidative stress. *Chem. Biol. Interact.* 95, 291-307.
- Hyttinen, J.M.T., Jansson, K., 1995. PM2 DNA damage induced by 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX). *Mutat. Res.* 348, 183-186.
- Hyttinen, J.M.T., Myöhänen, S., Jansson, K., 1996. Kinds of mutations induced by 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) in the *hprt* gene of Chinese hamster ovary cells. *Carcinogenesis* 17, 1179-1181.
- Hyttinen, J.M.T., Niittykoski, M., Jansson, K., 1995. Lack of uniformity in the mutational spectra of chlorohydroxyfuranones in *Salmonella typhimurium* strain TA100. *Mutagenesis* 10, 321-323.
- IARC, 1991. Chlorinated drinking-water, chlorination by-products, some other halogenated compounds, cobalt and cobalt compounds. IARC Monographs on the evaluation of carcinogenic risks to humans. Vol 52.

- IARC, 2004. Some drinking-water disinfectants and contaminants, including arsenic. Monographs on chloramine, chloral and chloral hydrate, dichloroacetic acid, trichloroacetic acid and 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone. IARC Monographs on the evaluation of carcinogenic risks to humans. Vol 84.
- Ishiguro, Y., LaLonde, R.T., Dence, C.W., 1987. Mutagenicity of chlorine-substituted furanones and their inactivation by reaction with nucleophiles. *Environ. Toxicol. Chem.* 6, 935-946.
- Ishiguro, Y., Santodonato, J., Neal, M.W., 1988. Mutagenic potency of chlorofuranones and related compounds in *Salmonella*. *Environ. Mol. Mutagen.* 11, 225-234.
- Jansson, K., 1998. Lack of induction of micronuclei in bone marrow erythrocytes of rats exposed to 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) for two years in a carcinogenicity bioassay. *Environ. Mol. Mutagen.* 32, 185-187.
- Jansson, K., Hyttinen, J.M.T., 1994. Induction of gene mutation in mammalian cells by 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX), a chlorine disinfection by-product in drinking water. *Mutat. Res.* 322, 129-132.
- Jansson, K., Hyttinen, J.M.T., Niittykoski, M., Mäki-Paakkanen, J., 1995. Mutagenicity in vitro of 3,4-dichloro-5-hydroxy-2(5H)-furanone (mucochloric acid), a chlorine disinfection by-product in drinking water. *Environ. Mol. Mutagen.* 25, 284-287.
- Jansson, K., Mäki-Paakkanen, J., Vaitinen, S.-L., Vartiainen, T., Komulainen, H., Tuomisto, J., 1993. Cytogenetic effects of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) in rat peripheral lymphocytes in vitro and in vivo. *Mutat. Res.* 229, 25-28.
- Kinae, N., Sugiyama, C., Nasuda, M.Y., Goto, K., Tokumoto, K., Furugori, M., Shimoi, K., 1992. Seasonal variation and stability of chlorinated organic mutagens in drinking water. *Wat. Sci. Tech.* 25, 333-340.
- King, T.J., Lampe, P.D., 2004. Mice deficient for the gap junction protein Connexin32 exhibit increased radiation-induced tumorigenesis associated with elevated mitogen-activated protein kinase (p44/Erk1, p42/Erk2) activation. *Carcinogenesis* 25, 669-680.
- Kitahori, Y., Naitoh, H., Konishi, N., Fukushima, M., Ohnishi, T., Hiasa, Y., 1996. G→A mutation of *ras* genes and infrequent *p53* gene mutation in rat transplantable thyroid carcinoma lines from tumors induced in vivo by *N*-bis(2-hydroxypropyl)nitrosamine. *Cancer Lett.* 100, 55-62.
- Kitahori, Y., Naitoh, H., Konishi, N., Ohnishi, T., Hiasa, Y., 1997. Genetic alterations in *N*-bis(2-hydroxypropyl)nitrosamine-induced rat transplantable thyroid carcinoma lines: analysis of the TSH-R, *Gαs*, *ras* and *p53* genes. *Carcinogenesis* 18, 265-269.
- Kitahori, Y., Naitoh, H., Konishi, N., Ohnishi, T., Shirai, T., Hiasa, Y., 1995. Frequent mutations of Ki-*ras* codon 12 in *N*-bis(2-hydroxypropyl)-nitrosamine-initiated thyroid, kidney and lung tumors in Wistar rats. *Cancer Lett.* 96, 155-161.
- Klaunig, J.E., Kamendulis, L.M., Xu, Y., 2000. Epigenetic mechanisms of chemical carcinogenesis. *Hum. Exp. Toxicol.* 19, 543-555.
- Knasmüller, S., Zöhrer, E., Kronberg, L., Kundi, M., Franzén, R., Schulte-Hermann, R., 1996. Mutational spectra of *Salmonella typhimurium* revertants induced by

- chlorohydroxyfuranones, byproducts of chlorine disinfection of drinking water. *Chem. Res. Toxicol.* 9, 374-381.
- Kobayashi, Y., Kawaoi, A., Katoh, R., 2002. Mutation of *ras* oncogene in di-isopropanolnitrosamine-induced rat thyroid carcinogenesis. *Virchows Arch.* 441, 289-295.
- Kojima, T., Mitaka, T., Mizuguchi, T., Mochizuki, Y., 1996. Effects of oxygen radical scavengers on connexins 32 and 26 expression in primary cultures of adult rat hepatocytes. *Carcinogenesis* 17, 537-544.
- Komulainen, H., Kosma, V.-M., Vaittinen, S.-L., Vartiainen, T., Kaliste-Korhonen, E., Lötjönen, S., Tuominen, R.K., Tuomisto, J., 1997. Carcinogenicity of the drinking water mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone in the rat. *J. Natl. Cancer Inst.* 89, 848-856.
- Komulainen, H., Tuominen, R.K., Kosma, V.-M., Huuskonen, H., 2000. 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX), a rat thyroid gland carcinogen, does not affect serum levels of TSH and thyroid hormones. *Environ. Toxicol. Pharmacol.* 8, 267-273.
- Koivusalo, M., Jaakkola, J.J.K., Vartiainen, T., Hakulinen, T., Karjalainen, S., Pukkala, E., Tuomisto, J., 1994. Drinking water mutagenicity and gastrointestinal and urinary tract cancers: An ecological study in Finland. *Am. J. Public Health* 84, 1223-1228.
- Koivusalo, M., Pukkala, E., Vartiainen, T., Jaakkola, J.J.K., Hakulinen, T., 1997. Drinking water chlorination and cancer – a historical cohort study in Finland. *Cancer Causes Control* 8, 192-200.
- Koivusalo, M., Vartiainen, T., Hakulinen, T., Pukkala, E., Jaakkola, J.J.K., 1995. Drinking water mutagenicity and leukemia, lymphomas, and cancers of the liver, pancreas, and soft tissue. *Arch. Environ. Health* 50, 269-276.
- Kolaja, K.J., Petrick, J.S., Klaassen, C.D., 2000. Inhibition of gap-junctional-intercellular communication in thyroid-follicular cells by propylthiouracil and low iodine diet. *Toxicology* 143, 195-202.
- Kronberg, L., Asplund, D., Mäki, J., Sjöholm, R., 1996. Reactions of mucochloric and mucobromic acid with adenosine and cytidine: Formation of chloro- and bromopropenal derivatives. *Chem. Res. Toxicol.* 9, 1257-1263.
- Kronberg, L., Franzén, R., 1993. Determination of chlorinated furanones, hydroxyfuranones, and butenedioic acids in chlorine-treated water and in pulp bleaching liquor. *Environ. Sci. Technol.* 27, 1811-1818.
- Kronberg, L., Holmbom, B., Reunanen, M., Tikkanen, L., 1988. Identification and quantification of the Ames mutagenic compound 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone and of its geometric isomer (*E*)-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid in chlorine-treated humic water and drinking water extracts. *Environ. Sci. Technol.* 22, 1097-1103.
- Kronberg, L., Karlsson, S., Sjöholm, R., 1993. Formation of ethenocarbaldehyde derivatives of adenosine and cytidine in reactions with mucochloric acid. *Chem. Res. Toxicol.* 6, 495-499.

- Kronberg, L., Sjöholm, R., Karlsson, S., 1992. Formation of 3,*N*<sup>4</sup>-ethenocytidine, 1,*N*<sup>6</sup>-ethenoadenosine, and 1,*N*<sup>2</sup>-ethenoguanosine in reactions of mucochloric acid with nucleosides. *Chem. Res. Toxicol.* 5, 852-855.
- Kronberg, L., Vartiainen, T., 1988. Ames mutagenicity and concentration of the strong mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone and of its geometric isomer E-2-chloro-3-(dichloromethyl)-4-oxo-butenoic acid in chlorine-treated tap waters. *Mutat. Res.* 206, 177-182.
- Laaksonen, M., Mäki-Paakkanen, J., Komulainen, H., 2001. Enhancement of 3-methylcholanthrene-induced neoplastic transformation by 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone in the two-stage transformation assay in C3H 10T1/2 cells. *Arch. Toxicol.* 75, 613-617.
- Laaksonen, M., Mäki-Paakkanen, J., Kronberg, L., Komulainen, H., 2003. Effects of chlorohydroxyfuranones on 3-methylcholanthrene-induced neoplastic transformation in the two-stage transformation assay in C3H 10T1/2 cells. *Arch. Toxicol.* 77, 594-600.
- LaLonde, R.T., Cook, G.P., Perakyla, H., Bu, L., 1991a. Structure-activity relationships of bacterial mutagens related to 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone: an emphasis on the effect of stepwise removal of chlorine from the dichloromethyl group. *Chem. Res. Toxicol.* 4, 540-545.
- LaLonde, R.T., Cook, G.P., Perakyla, H., Dence, C.W., 1991b. Effect on mutagenicity of the stepwise removal of hydroxyl group and chlorine atoms from 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone: <sup>13</sup>C NMR chemical shifts as determinants of mutagenicity. *Chem. Res. Toxicol.* 4, 35-40.
- LaLonde, R.T., Leo, H., Perakyla, H., Dence, C.W., Farrell, R.P., 1992. Associations of the bacterial mutagenicity of halogenated 2(5*H*)-furanones with their MNDO-PM3 computed properties and mode of reactivity with sodium borohydride. *Chem. Res. Toxicol.* 5, 392-400.
- LaLonde, R.T., Ramdayal, F., 1997. Mucochloric acid action on ΦX174 DNA: a comparison to other chlorine-substituted 2(5*H*)-furanones. *Chem. Res. Toxicol.* 10, 205-210.
- Lampe, P.D., TenBroek, E.M., Burt, J.M., Kurata, W.E., Johnson, R.G., Lau, A.F., 2000. Phosphorylation of connexin43 on serine368 by protein kinase C regulates gap junctional communication. *J. Cell Biol.* 149, 1503-1512.
- Lawrence, T.S., Beers, W.H., Gilula, N.B., 1978. Hormonal stimulation and cell communication in cocultures. *Nature* 272, 501-506.
- Le Curieux, F., Munter, T., Kronberg, L., 1997. Identification of adenine adducts formed in reaction of calf thymus DNA with mutagenic chlorohydroxyfuranones found in drinking water. *Chem. Res. Toxicol.* 10, 1180-1185.
- Le Curieux, F., Nessler, F., Munter, T., Kronberg, L., Marzin, D., 1999. Genotoxic activity of chlorohydroxyfuranones in the microscale micronucleus test on mouse lymphoma cells and the unscheduled DNA synthesis assay in rat hepatocytes. *Mutagenesis* 14, 457-462.

- Leithe, E., Cruciani, V., Sanner, T., Mikalsen, S.-O., Rivedal, E., 2003. Recovery of gap junctional intercellular communication after phorbol ester treatment requires proteasomal degradation of protein kinase C. *Carcinogenesis* 24, 1239-1245.
- Lemoine, N.R., Mayall, E.S., Williams, E.D., Thurston, V., Wynford-Thomas, D., 1988. Agent-specific *ras* oncogene activation in rat thyroid tumours. *Oncogene* 3, 541-544.
- Lemoine, N.R., Mayall, E.S., Wyllie, F.S., Williams, E.D., Goyns, M., Stringer, B., Wynford-Thomas, D., 1989. High frequency of *ras* oncogene activation in all stages of human thyroid tumorigenesis. *Oncogene* 4, 159-164.
- Loeb, K.R., Loeb, L.A., 2000. Significance of multiple mutations in cancer. *Carcinogenesis* 21, 379-385.
- Macaluso, M., Russo, G., Cinti, C., Bazan, V., Gebbia, N., Russo, A., 2002. Ras family genes: an interesting link between cell cycle and cancer. *J. Cell. Physiol.* 192, 125-130.
- Mally, A., Chipman, J.K., 2002. Non-genotoxic carcinogens: early effects on gap junctions, cell proliferation and apoptosis in the rat. *Toxicology* 180, 233-248.
- Malumbres, M., Pellicer, A., 1998. RAS pathways to cell cycle control and cell transformation. *Front. Biosci.* 3, d887-912.
- Marsteinstredet, U., Brunborg, G., Bjørås, M., Söderlund, E., Seeberg, E., Kronberg, L., Holme, J.A., 1997a. DNA damage induced by 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX) in HL-60 cells and purified DNA in vitro. *Mutat. Res.* 390, 171-178.
- Marsteinstredet, U., Wiger, R., Brunborg, G., Hongslo, J.K., Holme, J.A., 1997b. Apoptosis in HL-60 cells induced by 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX). *Chem. Biol. Interact.* 106, 89-107.
- Masui, T., Nakanishi, H., Inada, K.-i., Imai, T., Mizoguchi, Y., Yada, H., Futakuchi, M., Shirai, T., Tatematsu, M., 1997. Highly metastatic hepatocellular carcinomas induced in male F344 rats treated with *N*-nitrosomorpholine in combination with other hepatocarcinogens show a high incidence of *p53* gene mutations along with altered mRNA expression of tumor-related genes. *Cancer Lett.* 112, 33-45.
- Matesic, D.F., Rupp, H.L., Bonney, W.J., Ruch, R.J., Trosko, J.E., 1994. Changes in gap-junction permeability, phosphorylation, and number mediated by phorbol ester and non-phorbol-ester tumor promoters in rat liver epithelial cells. *Mol. Carcinog.* 10, 226-236.
- Matsumoto, K., Iwase, T., Hirono, I., Nishida, Y., Iwahori, Y., Hori, T., Asamoto, M., Takasuka, N., Kim, D.J., Ushijima, T., Nagao, M., Tsuda, H., 1997. Demonstration of *ras* and *p53* gene mutations in carcinomas in the forestomach and intestine and soft tissue sarcomas induced by *N*-methyl-*N*-nitrosourea in the rat. *Jpn. J. Cancer Res.* 88, 129-136.
- Matsumura, H., Watanabe, M., Matsumoto, K., Ohta, T., 1994. 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) induces gene mutations and inhibits metabolic cooperation in cultured chinese hamster cells. *J. Toxicol. Environ. Health* 43, 65-72.

- McClain, R.M., 1995. Mechanistic considerations for the relevance of animal data on thyroid neoplasia to human risk assessment. *Mutat. Res.* 333, 131-142.
- McDonald, T.A., Komulainen, H., 2005. Carcinogenicity of the chlorination disinfection by-product MX. *J. Environ. Sci. Health Part C Environ. Carcinog. Ecotoxicol. Rev.* 23, 163-214.
- Meda, P., Pepper, M.S., Traub, O., Willecke, K., Gros, D., Beyer, E., Nicholson, B., Paul, D., Orci, L., 1993. Differential expression of gap junction connexins in endocrine and exocrine glands. *Endocrinology* 133, 2371-2378.
- Meier, J.R., Blazak, W.F., Knohl, R.B., 1987a. Mutagenic and clastogenic properties of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone: a potent bacterial mutagen in drinking water. *Environ. Mol. Mutagen.* 10, 411-424.
- Meier, J.R., Knohl, R.B., Coleman, W.E., Ringhand, H.P., Munch, J.W., Kaylor, W.H., Streicher, R.P., Kopfler, F.C., 1987b. Studies on the potent bacterial mutagen, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone: aqueous stability, XAD recovery and analytical determination in drinking water and in chlorinated humic acid solutions. *Mutat. Res.* 189, 363-373.
- Meier, J.R., Monarca, S., Patterson, K.S., Villarini, M., Daniel, F.B., Moretti, M., Pasquini, R., 1996. Urine mutagenicity and biochemical effects of the drinking water mutagen, 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX), following repeated oral administration to mice and rats. *Toxicology* 110, 59-70.
- Meier, J.R., Ringhand, H.P., Coleman, W.E., Schenck, K.M., Munch, J.W., Streicher, R.P., Kaylor, W.H., Kopfler, F.C., 1986. Mutagenic by-products from chlorination of humic acid. *Environ. Health Perspect.* 69, 101-107.
- Miller, M.S., 1999. Tumor suppressor genes in rodent lung carcinogenesis: mutation of *p53* does not appear to be an early lesion in lung tumor pathogenesis. *Toxicol. Appl. Pharmacol.* 156, 70-77.
- Mizukami, Y., Nonomura, A., Michigishi, T., Noguchi, M., Nakamura, S., Hashimoto, T., 1995. Differential (Ha-, K- and N-) ras p21 expression in benign and malignant human thyroid tumors: an immunohistochemical study. *Anticancer Res.* 15, 755-759.
- Morris, R.D., 1995. Drinking water and cancer. *Environ. Health Perspect.* 103 Suppl 8:225-231.
- Morris, R.D., Audet, A.-M., Angelillo, I.F., Chalmers, T.C., Mosteller, F., 1992. Chlorination, chlorination by-products, and cancer: A meta-analysis. *Am. J. Public Health* 82, 955-963.
- Motoi, N., Sakamoto, A., Yamochi, T., Horiuchi, H., Motoi, T., Machinami, R., 2000. Role of ras mutation in the progression of thyroid carcinoma of follicular epithelial origin. *Pathol. Res. Pract.* 196, 1-7.
- Movsesyan, V.A., Yakovlev, A.G., Dabaghyan, E.A., Stoica, B.A., Faden, A.I., 2002. Ceramide induces neuronal apoptosis through the caspase-9/caspase-3 pathway. *Biochem. Biophys. Res. Commun.* 299, 201-207.

- Munter, T., Kronberg, L., Sjöholm, R., 1996. Identification of adducts formed in reaction of adenosine with 3-chloro-4-methyl-5-hydroxy-2(5*H*)-furanone, a bacterial mutagen present in chlorine disinfected drinking water. *Chem. Res. Toxicol.* 9, 703-708.
- Munter, T., Le Curieux, F., Sjöholm, R., Kronberg, L., 1998. Reaction of the potent bacterial mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) with 2'-deoxyadenosine and calf thymus DNA: Identification of fluorescent propenoformyl derivatives. *Chem. Res. Toxicol.* 11, 226-233.
- Munter, T., Le Curieux, F., Sjöholm, R., Kronberg, L., 1999a. Identification of an ethenoformyl adduct formed in the reaction of the potent bacterial mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone with guanosine. *Chem. Res. Toxicol.* 12, 46-52.
- Munter, T., Le Curieux, F., Sjöholm, R., Kronberg, L., 1999b. Identification of adducts formed in reactions of 2'-deoxyadenosine and calf thymus DNA with the bacterial mutagen 3-chloro-4-(chloromethyl)-5-hydroxy-2(5*H*)-furanone. *Chem. Res. Toxicol.* 12, 1205-1212.
- Mäki-Paakkanen, J., Jansson, K., 1995. Cytogenetic effects in the peripheral lymphocytes and kidney cells of rats exposed to 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) orally on three consecutive days. *Mutat. Res.* 343, 151-156.
- Mäki-Paakkanen, J., Jansson, K., Vartiainen, T., 1994. Induction of mutation, sister-chromatid exchanges, and chromosome aberrations by 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone in Chinese hamster ovary cells. *Mutat. Res.* 310, 117-123.
- Mäki-Paakkanen, J., Komulainen, H., Kronberg, L., 2004. Bacterial and mammalian-cell genotoxicity of mixtures of chlorohydroxyfuranones, by-products of water chlorination. *Environ. Mol. Mutagen.* 43, 217-225.
- Mäki-Paakkanen, J., Laaksonen, M., Munter, T., Kronberg, L., Komulainen, H., 2001. Comparable DNA and chromosome damage in Chinese hamster ovary cells by chlorohydroxyfuranones. *Environ. Mol. Mutagen.* 38, 297-30.
- Nakajima, M., Shimada, S., Nagai, M., Mizuhashi, F., Sugiyama, C., Masuda, S., Hayashi, M., Kinae, N., 2005. 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone [MX] shows initiating and promoting activities in a two-stage BALB/c 3T3 cell transformation assay. *Mutagenesis* 20, 375-390.
- Neveu, M.J., Hully, J.R., Babcock K.L., Vaughan, J., Hertzberg, E.L., Nicholson, B.J., Paul, D.L., Pitot, H.C., 1995. Proliferation-associated differences in the spatial and temporal expression of gap junction genes in rat liver. *Hepatology* 22, 202-212.
- Niittykoski, M., Hyttinen, J.M.T., Jansson, K., 1995. Salmonella and mammalian-cell mutagenicity of 3-chloro-4-(chloromethyl)-5-hydroxy-2(5*H*)-furanone. *Mutat. Res.* 348, 51-55.
- Nishikawa, A., Furukawa, F., Lee, I.-S., Kasahara, K.-i., Tanakamaru, Z.-y., Nakamura, H., Miyauchi, M., Kinae, N., Hirose, M., 1999. Promoting effects of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone on rat glandular stomach carcinogenesis initiated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Cancer Res.* 59, 2045-2049.

- Nishikawa, A., Kinae, N., Furukawa, F., Mitsui, M., Enami, T., Hasegawa, T., Takahashi, M., 1994. Enhancing effects of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) on cell proliferation and lipid peroxidation in the rat gastric mucosa. *Cancer Lett.* 85, 151-157.
- Nishikawa, A., Sai, K., Okazaki, K., Son H.Y., Kanki, K., Nakajima, M., Kinae, N., Nohmi, T., Trosko, J.E., Inoue, T., Hirose, M., 2006. MX, a by-product of water chlorination, lacks in vivo genotoxicity in *gpt* delta mice but inhibits gap junctional intercellular communication in rat WB cells. *Environ. Mol. Mutagen.* 47, 48-55.
- Nunn, J.W., Chipman, J.K., 1994. Induction of DNA strand breaks by 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone and humic substances in relation to glutathione and calcium status in human white blood cells. *Mutat. Res.* 341, 133-140.
- Nunn, J.W., Davies, J.E., Chipman, J.K., 1997. Production of unscheduled DNA synthesis in rodent hepatocytes in vitro, but not in vivo, by 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX). *Mutat. Res.* 373, 67-73.
- Nyska, A., Moomaw, C.R., Foley, J.F., Maronpot, R.R., Malarkey, D.E., Cummings, C.A., Peddada, S., Moyer, C.F., Allen, D.G., Travlos, G., Chan, P.C., 2002. The hepatic endothelial carcinogen riddelliine induces endothelial apoptosis, mitosis, S phase, and p53 and hepatocytic vascular endothelial growth factor expression after short-term exposure. *Toxicol. Appl. Pharmacol.* 184, 153-164.
- Perantoni, A.O., Rice, J.M., 1999. Mutation patterns in non-*ras* oncogenes and tumour suppressor genes in experimentally induced tumours. In: McGregor, D.B, Rice, J.M., Venitt, S. (eds.), *The use of short- and medium-term tests for carcinogens and data on genetic effects in carcinogenic hazard evaluation*, IARC Scientific Publications No. 146, IARC, Lyon, pp. 87-122.
- Pesse, B., Levrard, S., Feihl, F., Waeber, B., Gavillet, B., Pacher, P., Liaudet, L., 2005. Peroxynitrite activates ERK via Raf-1 and MEK, independently from EGF receptor and p21<sup>Ras</sup> in H9C2 cardiomyocytes. *J. Mol. Cell. Cardiol.* 38, 765-775.
- Pitot, H.C., 2001. Pathways of progression in hepatocarcinogenesis. *Lancet* 358, 859-860.
- Pitot, H.C., 1993. The molecular biology of carcinogenesis. *Cancer* 72, 962-970.
- Pitot, H.C., Dragan, Y., 1991. Facts and theories concerning the mechanisms of carcinogenesis. *FASEB J.* 5, 2280-2286.
- Pitts, J.D., Finbow, M.E., 1986. The gap junction. *J. Cell. Sci. (Suppl.)* 4, 239-266.
- Pluquet, O., Hainaut, P., 2001. Genotoxic and non-genotoxic pathways of p53 induction. *Cancer Lett.* 174, 1-15.
- Poirier, M.C., 2004. Chemical-induced DNA damage and human cancer risk. *Nat. Rev. Cancer* 4, 630-637.
- Richardson, S.D., Simmons, J.E., Rice, G., 2002. Disinfection byproducts: the next generation. *Environ. Sci. Technol.* 36, 198A-205A.
- Rivedal, E., Leithe, E., 2005. Connexin43 synthesis, phosphorylation, and degradation in regulation of transient inhibition of gap junction intercellular communication by the phorbol ester TPA in rat liver epithelial cells. *Exp. Cell Res.* 302, 143-152.

- Rivedal, E., Opsahl, H., 2001. Role of PKC and MAP kinase in EGF- and TPA-induced connexin43 phosphorylation and inhibition of gap junctional intercellular communication in rat liver epithelial cells. *Carcinogenesis* 22, 1543-1550.
- Rodriguez-Viciana, P., Sabatier, C., McCormick, F., 2004. Signaling specificity by Ras family GTPases is determined by the full spectrum of effectors they regulate. *Mol. Cell. Biol.* 24, 4943-4954.
- Romero, J., Ventura, F., Caixach, J., Rivera, J., Guerrero, R., 1997. Identification and quantification of the mutagenic compound 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) in chlorine-treated water. *Bull. Environ. Contam. Toxicol.* 59, 715-722.
- Ruch, R.J., Bonney, W.J., Sigler, K., Guan, X., Matesic, D., Schafer, L.D., Dupont, E., Trosko, J.E., 1994. Loss of gap junctions from DDT-treated rat liver epithelial cells. *Carcinogenesis* 15, 301-306.
- Ruch, R.J., Trosko, J.E., Madhukar, B.V., 2001. Inhibition of connexin43 gap junctional intercellular communication by TPA requires ERK activation. *J. Cell. Biochem.* 83, 163-169.
- Ryan, K.M., Phillips, A.C., Vousden, K.H., 2001. Regulation and function of the p53 tumor suppressor protein. *Curr. Opin. Cell Biol.* 13, 332-337.
- Saez, J.C., Conner, J.A., Spray, D.C., Bennett, M.V.L., 1989. Hepatocyte gap junctions are permeable to a second messenger, inositol 1,4,5-triphosphate and to calcium ions. *Proc. Natl. Acad. Sci. USA* 86, 2708-2712.
- Sai, K., Upham, B.L., Kang, K.-S., Hasegawa, R., Inoue, T., Trosko, J.E., 1998. Inhibitory effect of pentachlorophenol on gap junctional intercellular communication in rat liver epithelial cell in vitro. *Cancer Lett.* 130, 9-17.
- Sasaki, Y.F., Nishidate, E., Izumiyama, F., Watanabe-Akanuma, M., Kinae, N., Matsusaka, N., Tsuda, S., 1997. Detection of in vivo genotoxicity of 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX) by the alkaline single cell gel electrophoresis (Comet) assay in multiple mouse organs. *Mutat. Res.* 393, 47-53.
- Shaughnessy, D.T., Ohe, T., Landi, S., Warren, S.H., Richard, A.M., Munter, T., Franzén, R., Kronberg, L., DeMarini, D.M., 2000. Mutation spectra of the drinking water mutagen 3-chloro-4-methyl-5-hydroxy-2(5H)-furanone (MCF) in *Salmonella* TA100 and TA104: comparison to MX. *Environ. Mol. Mutagen.* 35, 106-113.
- Sills, R.C., Boorman, G.A., Neal, J.E., Hong, H.L., Devereux, T.R., 1999. Mutations in *ras* genes in experimental tumours of rodents. In: McGregor, D.B., Rice, J.M., Venitt, S. (eds.), *The use of short- and medium-term tests for carcinogens and data on genetic effects in carcinogenic hazard evaluation*, IARC Scientific Publications No. 146, IARC, Lyon, pp. 55-71.
- Simpson, K.L., Hayes, K.P., 1998. Drinking water disinfection by-products: an Australian perspective. *Wat. Res.* 32, 1522-1528.
- Singh, A., Sowjanya, A.P., Ramakrishna, G., 2005. The wild-type Ras: road ahead. *FASEB J.* 19, 161-169.

- Smeds, A., Vartiainen, T., Mäki-Paakkanen, J., Kronberg, L., 1997. Concentrations of Ames mutagenic chlorohydroxyfuranones and related compounds in drinking waters. *Environ. Sci. Technol.* 31, 1033-1039.
- Son, H.-Y., Nishikawa, A., Ikeda, T., Furukawa, F., Hirose, M., 2000a. Lack of modification by environmental estrogenic compounds of thyroid carcinogenesis in ovariectomized rats pretreated with *N*-bis(2-hydroxypropyl)nitrosamine (DHPN). *Jpn. J. Cancer Res.* 91, 966-972.
- Son, H.-Y., Nishikawa, A., Ikeda, T., Nakamura, H., Miyauchi, M., Imazawa, T., Furukawa, F., Hirose, M., 2000b. Lack of modifying effects of environmental estrogenic compounds on the development of thyroid proliferative lesions in male rats pretreated with *N*-bis(2-hydroxypropyl)nitrosamine (DHPN). *Jpn. J. Cancer Res.* 91, 899-905.
- Stanley, L.A., 1995. Molecular aspects of chemical carcinogenesis: The roles of oncogenes and tumour suppressor genes. *Toxicology* 96, 173-194.
- Steffensen, I.-L., Paulsen, J.E., Engeset, D., Kronberg, L., Alexander, J., 1999. The drinking water chlorination by-products 3,4-dichloro-5-hydroxy-2[5*H*]-furanone (mucochloric acid) and 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5*H*]-furanone do not induce preneoplastic or neoplastic intestinal lesions in F344 rats, Balb/cA mice or C57BL/6J-*Min* mice. *Pharmacol. Toxicol.* 85, 56-64.
- Stevenson, M.A., Pollock, S.S., Coleman, C.N., Calderwood, S.K., 1994. X-irradiation, phorbol esters, and H<sub>2</sub>O<sub>2</sub> stimulate mitogen-activated protein kinase activity in NIH-3T3 cells through the formation of reactive oxygen intermediates. *Cancer Res.* 54, 12-15.
- Strauss, B.S., 1991. The 'A' rule of mutagen specificity: a consequence of DNA polymerase bypass of non-instructional lesions? *Bioessays* 13, 79-84.
- Suarez, H.G., du Villard, J.A., Severino, M., Caillou, B., Schlumberger, M., Tubiana, M., Parmentier, C., Monier, R., 1990. Presence of mutations in all three *ras* genes in human thyroid tumors. *Oncogene* 5, 565-570.
- Suzuki, J., Na, H.K., Upham, B.L., Chang, C.C., Trosko, J.E., 2000.  $\lambda$ -carrageenan-induced inhibition of gap-junctional intercellular communication in rat liver epithelial cells. *Nutr. Cancer* 36, 122-128.
- Suzuki, N., Nakanishi, J., 1990. The determination of strong mutagen, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone in drinking water in Japan. *Chemosphere* 21, 387-392.
- Takahashi, M., Wakabayashi, K., 2004. Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. *Cancer Sci.* 95, 475-480.
- Thomas, G.A., Williams, E.D., 1991. Evidence for and possible mechanisms of non-genotoxic carcinogenesis in the rodent thyroid. *Mutat. Res.* 248, 357-370.
- Thompson, N., Lyons, J., 2005. Recent progress in targeting the Raf/MEK/ERK pathway with inhibitors in cancer drug discovery. *Curr. Opin. Pharmacol.*
- Tikkanen, L., Kronberg, L., 1990. Genotoxic effects of various chlorinated butenoic acids identified in chlorinated drinking water. *Mutat. Res.* 240, 109-116.

- Trosko, J.E., 1997. Challenge to the simple paradigm that 'carcinogens' are 'mutagens' and to the in vitro and in vivo assays used to test the paradigm. *Mutat. Res.* 373, 245-249.
- Trosko, J.E., 2001. Commentary: Is the concept of "tumor promotion" a useful paradigm? *Mol. Carcinog.* 30, 131-137.
- Trosko, J.E., Chang, C.C., 1988. Nongenotoxic mechanisms in carcinogenesis: Role of inhibited intercellular communication. In: Hart, R.W., Hoerger, F.D. (eds.), *Banbury Report 31: Carcinogen Risk Assessment: New Directions in the Qualitative and Quantitative Aspects*. Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., pp. 139-171.
- Trosko, J.E., Chang, C.C., Upham, B.L., Wilson, M., 1998. Epigenetic toxicology as toxicant-induced changes in intracellular signaling leading to altered gap junctional intercellular communication. *Toxicol. Lett.* 102-103, 71-78.
- Trosko, J.E., Chang, C.C., Wilson, M.R., Upham, B., Hayashi, T., Wade, M., 2000. Gap junctions and the regulation of cellular functions of stem cells during development and differentiation. *Methods* 20, 245-264.
- Trosko, J.E., Goodman, J.I., 1994. Intercellular communication may facilitate apoptosis: implications for tumor promotion. *Mol. Carcinog.* 11, 8-12.
- Trosko, J.E., Ruch, R.J., 1998. Cell-cell communication in carcinogenesis. *Front. Biosci.* 3, D208-D236.
- Trosko, J.E., Upham, B.L., 2005. The emperor wears no clothes in the field of carcinogen risk assessment: ignored concepts in cancer risk assessment. *Mutagenesis* 20, 81-92.
- Tuppurainen, K., 1992. On the electronic structure of MX compounds. *J. Mol. Struct. (Theochem)* 276, 299-306.
- Tuppurainen, K., 1997. A plausible mechanism for the mutagenic activity (*Salmonella typhimurium* TA100) of MX compounds: a formation of CG-CG<sup>+</sup>-CG radical cation by one-electron reduction. *SAR QSAR Environ. Res.* 7, 281-286.
- Tuppurainen, K., Lötjönen, S., 1993. On the mutagenicity of MX compounds. *Mutat. Res.* 287, 235-241.
- Tuppurainen, K., Lötjönen, S., Laatikainen, R., Vartiainen, T., 1992. Structural and electronic properties of MX compounds related to TA100 mutagenicity. A semi-empirical molecular orbital QSAR study. *Mutat. Res.* 266, 181-188.
- Tuppurainen, K., Lötjönen, S., Laatikainen, R., Vartiainen, T., Maran, U., Strandberg, M., Tamm, T., 1991. About the mutagenicity of chlorine-substituted furanones and halopropenals. A QSAR study using molecular orbital indices. *Mutat. Res.* 247, 97-102.
- Upham, B.L., Kang, K.S., Cho, H.Y., Trosko, J.E., 1997. Hydrogen peroxide inhibits gap junctional intercellular communication in glutathione sufficient but not glutathione deficient cells. *Carcinogenesis* 18, 37-42.
- U.S. Environmental Protection Agency (US EPA), 2002. The occurrence of disinfection by-products (DBPs) of health concern in drinking water: Results of a nationwide DBP

- occurrence study. Athens, G.A.: National Exposure Research Laboratory, Office of Research and Development, US EPA.
- Vancutsem, P.M., Lazarus, P., Williams, G.M., 1994. Frequent and specific mutations of the rat p53 gene in hepatocarcinomas induced by tamoxifen. *Cancer Res.* 54, 3864-3867.
- van Gijssel, H.E., Maassen, C.B.M., Mulder, G.J., Meerman, J.H.N., 1997. p53 protein expression by hepatocarcinogens in the rat liver and its potential role in mitoinhibition of normal hepatocytes as a mechanism of hepatic tumour promotion. *Carcinogenesis* 18, 1027-1033.
- Vartiainen, T., Heiskanen, K., Lötjönen, S., 1991. Analysis and some chemical properties of MX (3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone), the potent drinking water mutagen. *Fresenius J. Anal. Chem.* 340, 230-233.
- Vartiainen, T., Lampelo, S., Tuomisto, J., Lötjönen, S., 1989. The strong mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) of drinking water is drastically metabolized by placental S9 and rat liver S9 with and without vitamin C. *Mutat. Res.* 216, 282-283.
- Villanueva, C.M., Cantor, K.P., Cordier, S., Jaakkola, J.J.K., King, W.D., Lynch, C.F., Porru, S., Kogevinas, M., 2004. Disinfection byproducts and bladder cancer: a pooled analysis. *Epidemiology* 15, 357-367.
- Wartell, R.M., Hosseini, S., Powell, S., Zhu, J., 1998. Detecting single base substitutions, mismatches and bulges in DNA by temperature gradient gel electrophoresis and related methods. *J. Chromatogr. A* 806, 169-185.
- Watanabe-Akanuma, M., Ohta, T., 1994. Effects of DNA repair deficiency on the mutational specificity in the *lacZ* gene of *Escherichia coli*. *Mutat. Res.* 311, 295-304.
- Watanabe, M., Kobayashi, H., Ohta, T., 1994. Rapid inactivation of 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX), a potent mutagen in chlorinated drinking water, by sulfhydryl compounds. *Mutat. Res.* 312, 131-138.
- Wilson, M.R., Close, T.W., Trosko, J.E., 2000. Cell population dynamics (apoptosis, mitosis, and cell-cell communication) during disruption of homeostasis. *Exp. Cell Res.* 254, 257-268.
- Wirnitzer, U., Töpfer, R., Rosenbruch, M., 1998. Altered p53 expression in early stages of chemically induced rodent hepatocarcinogenesis. *Toxicol. Pathol.* 26, 636-645.
- Woodruff, N.W., Durant, J.L., Donhoffner, L.L., Penman, B.W., Crespi, C.L., 2001. Human cell mutagenicity of chlorinated and unchlorinated water and the disinfection byproduct 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX). *Mutat. Res.* 495, 157-168.
- Wright, J.M., Schwartz, J., Vartiainen, T., Mäki-Paakkanen, J., Altshul, L., Harrington, J.J., Dockery, D.W., 2002. 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) and mutagenic activity in Massachusetts drinking water. *Environ. Health Perspect.* 110, 157-164.
- Yamanoshita, O., Kubota, T., Hou, J., Ping, Y.-M., Zhang, X.-L., Li, X.-P., Li, S.-S., Li, X.-X., Zhu, D.-C., Fukushima, Y., Nakajima, T., 2005. DHPLC is superior to SSCP in screening p53 mutations in esophageal cancer tissues. *Int. J. Cancer* 114, 74-79.

- Yamasaki, H., Naus, C.C.G., 1996. Role of connexin genes in growth control. *Carcinogenesis* 17, 1199-1213.
- Yotti, L.P., Chang, C.C, Trosko, J.E., 1979. Elimination of metabolic cooperation in Chinese hamster cells by a tumor promoter. *Science* 206, 1089-1091.
- Zeni, O., Salvemini, F., Di Pietro, R., Buonincontri, D., Komulainen, H., Romanò, M., Scarfi, M.R., 2004. Induction of oxidative stress in murine cell lines by 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX). *Toxicol. Lett.* 147, 79-85.
- Zou, H., Xu, X., Zhang, J., Zhu, Z., 1995. The determination of strong mutagen MX [3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone] in drinking water in China. *Chemosphere* 30, 2219-2225.
- Zuidervaart, W., van Nieuwpoort, F., Stark, M., Dijkman, R., Packer, L., Borgstein, A.M., Pavey, S., van der Velden, P., Out, C., Jager, M.J., Hayward, N.K., Gruis, N.A., 2005. Activation of the MAPK pathway is a common event in uveal melanomas although it rarely occurs through mutation of BRAF or RAS. *Br. J. Cancer* 92, 2032-2038.