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Research Article

Genotoxicity Analysis of Two Hydroxyfuranones, Byproducts of Water Disinfection, in Human Cells Treated In Vitro

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In general, water for human consumption is chemically disinfected, usually by adding chlorine. As well as producing safe drinking water however, the chlorine treatment, also results in a number of disinfection byproducts (DBPs). One important class of these DBPs is made up of hydroxyfuranones (HFs). In this article, we report the results of a recent investigation to assess the genotoxicity of two HFs, namely mucobromic acid (MBA) and mucochloric acid (MCA), in cultured human cells. The comet assay is used to measure the induction of primary DNA damage and to determine the DNA repair kinetics and the ability of the tested compounds to cause oxidative damage. In addition, the micronucleus (MN) assay is applied to evaluate chromosome damage. The results of the comet assay reveal that both HFs are clearly genotoxic leading to high levels of DNA breaks, and that MBA is more effective than MCA. According to the comet results, the DNA damage induced by MBA repairs well over time, but not the one induced by MCA. Furthermore, HFs produce high levels of oxidized bases. In contrast, the results from the MN assay, which measures the induction of clastogenic and/or aneugenic effects, are mainly negative for the two HFs tested, although MCA is able to increase significantly the frequency of micronuclei in binucleated TK cells, at the concentration of 10 μM. Environ. Mol. Mutagen. 50:413–420, 2009.

Key words: disinfection byproducts; hydroxyfuranones; genotoxicity; comet assay; micronucleus assay

INTRODUCTION

Chemical treatment is the most common means of disinfecting drinking water, but a number of different disinfection byproducts (DBPs) are formed during the chlorination process, which could be considered important environmental hazardous chemicals because of the long-term implications for human health [Arbuckle et al., 2002; Komulainen, 2004]. DBPs are produced during the chlorination process in the treatment plants by the reaction of chlorine with the organic and inorganic matter present in raw water. Hundreds of different DBPs may be formed, depending on the amount of organic matter and the dose of chlorine used. In addition, other factors can contribute to the generation of different kinds of DBPs, such as the use of secondary disinfectants, pH, and temperature [Miller and Uden, 1983; Reckhow et al., 1990; Zhang et al., 2000; Nissinen et al., 2002; Nikolaou et al., 2004; Hua et al., 2006]. If the raw water contains bromide or iodide ions, brominated or iodinated byproducts are formed [Richardson et al., 2007].

The first group of DBPs described was the trihalomethanes (THMs) [Rook, 1974]. To date, more than 600 DBPs have been reported in the literature [Richardson, 1998], but only a small amount have been evaluated for genotoxic or mutagenic potential and possible adverse health effects. It is worth remembering that more than

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60% of the chlorinated DBPs are considered unknown organic halogens, the so-called unidentified DBPs.

Among DBPs, the THMs (chloroform, dichlorobromomethane, chlorodibromomethane, and bromoform) are the most studied, being also the main group detected in drinking water. Another group that has deserved much attention is the one formed by 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone, known as Mutagen X or MX, and their structural analogues. MX is one of the most potent direct-acting mutagens ever tested in the Ames strain TA100, and it is responsible for at least one-third of the total mutagenicity detected in chlorine-disinfected drinking water [Meier et al., 1987; Fekadu et al., 1994].

In the group of chlorohydroxyfuranones (CHFs), there are also other compounds, but with less mutagenic potency than MX. One of them is the mucochloric acid (MCA, 3,4-dichloro-5-hydroxy-2(5H)-furanone), which has been identified in drinking water at approximately the same concentration of MX [Kronberg and Franzen, 1993; Smeds et al., 1997]. Because of the positive results of CHFs in several mutagenicity assays and their similar structure to the MX, they have raised concern about their potential health hazards. Thus, all CHFs, including the MCA, have been incorporated by scientists of the U.S. Environmental Protection Agency in the list of priority DBPs for a Nationwide Occurrence Study [Krasner et al., 2006]. One HF that has not been identified in drinking water is the mucobromic acid (MBA, 3,4-dibromo-5-hydroxy-2(5H)-furanone), brominated analogue of MCA, but it is believed to be present especially in surface water with high amounts of bromide. Therefore, the aim of this study was to evaluate the genotoxic potential of two HFs, namely MBA and MCA, in cultured human cells.

These HFs have been tested using two genotoxicity assays. One is the single-cell gel electrophoresis (SCGE) assay or comet test, which is a very simple, rapid, and sensitive technique for measuring DNA damage [Fairbairn et al., 1995]. It can detect DNA damage frequencies of less than 1 in 10^7 bases [Lacoste et al., 2006], DNA single and double-strand breaks, incomplete excision repair, and alkali-labile sites.

With the comet assay, oxidative DNA damage can also be analyzed, by using enzymes detecting this kind of damage, such as formamidopyrimidine DNA glycosilase (fpg) and endonuclease III (endo III). These repair enzymes break the DNA at sites with oxidized purines and pyrimidines, respectively [Collins, 2005]. Furthermore, by measuring the remaining unrepaired DNA over time by means of repair kinetics experiments, it can be determined whether the cells can repair the induced lesions and how rapid the repair process is [Collins et al., 1995; Collins, 2004; Lacoste et al., 2007].

The second assay used is the micronucleus (MN) test. With this assay, other types of effects are evaluated corresponding to the chromosome damage induced by double-strand breaks or by aneuploidy [Fenech et al., 1999; Decordier and Kirsch-Volders, 2006]. Both genotoxic effects are of great relevance in carcinogenesis. It should be mentioned that a recent study has shown the usefulness of the MN assay as a surrogate biomarker of cancer risk [Bonassi et al., 2007].

The combined use of the indicated experimental approaches enables us to obtain new information on the characteristics of the DNA damage induced in human cells by the selected HFs (MBA and MCA).

MATERIALS AND METHODS

TK6 and Lymphocyte Cultures

The human lymphoblastoid TK6 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). This cell line has been used extensively for mutagenicity and other genotoxicity studies, including both the comet and the MN assays [McNamee et al., 2000; Guillamet et al., 2004; Ferrara et al., 2006; Mishima et al., 2008].

TK6 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Lymphocyte cultures were set up by adding 0.5 mL of heparinized whole blood, from one young nonsmoking healthy male donor, in 4.5 mL RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 1% phytohaemagglutinin, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Chemicals

Mucobromic acid (MBA, $C_4H_2Br_2O_3$) (CAS 488-11-9, 99% purity) and mucochloric acid (MCA, $C_4H_2Cl_2O_3$) (CAS 87-56-9, 99% purity), as well as mitomycin C (MMC) and hydrogen peroxide (H_2O_2) used for positive controls were purchased from Sigma-Aldrich (St Louis, MO).

Professor A. Collins (Institute for Nutrition Research, University of Oslo, Norway) kindly provided endonuclease III (endo III) and formamidopyrimidine glycosylase (fpg).

Comet Assay

TK6 cell cultures were centrifuged at 500*g* for 2 min, and the pellet was resuspended in RPMI 1640 medium (10^6 cell in 1 mL). Each DBP was dissolved in distilled water, and five or six concentrations of each DBP were tested. Aliquots of 10 μL of each solution were added to the cultures for 3 hr at 37°C. An aliquot of 10 μL H_2O_2 (2,000 μM) was included as positive control, and 10 μL of vehicle (dimethyl sulfoxide, DMSO) was used as negative control. Cell viability was evaluated as quickly as possible, with a mix of fluorescein diacetate (FDA) and ethidium bromide (EtBr) [Strauss, 1991]. Two hundred cells were scored for viability in each treatment. More than 70% of cells were viable in the applied treatments, which agree with the conditions required in the comet assay [Henderson et al., 1998]. The assay was performed as previously described by Singh et al. [1988] with minor modifications. Approximately 40,000 cells in 20 μL were carefully resuspended in 75 μL of 0.5% low-melting-point agarose (LMA), layered onto microscope slides precoated with 150 μL of 0.5% normal-melting-point agarose (NMA) (dried at 65°C), and covered with a coverslip and kept at 4°C until solidification. Then, coverslips were removed and cells were lysed for 2 hr at 4°C in a dark chamber containing a cold fresh lysing solution. To allow DNA denaturation, unwinding, and exposure of alkali-labile

sites, slides were placed for 40 min in a horizontal gel electrophoresis tank filled with freshly cold electrophoresis solution. Electrophoresis was performed in the same buffer for 20 min at 0.73 V/cm and 300 mA. Next, slides were neutralized with two 5-min washes with 0.4 M Tris (pH 7.5), fixed with absolute ethanol for 3 min, and stored in the dark at room temperature until scoring. Just before microscopic analysis, the slides were stained with 60 μL of EtBr (0.4 μg/mL). The images were examined at 400× magnification with a Komet 5.5 image analysis system (Kinetic Imaging, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope equipped with a 480–550 nm wide band excitation filter and a 590 nm barrier filter. One hundred randomly selected cells (50 from each of the two replicate slides) were analyzed per sample. The percentage of DNA in the tail was used to evaluate DNA damage and computed using the Komet version 5.5 software.

DNA Damage Repair Analysis

To determine how DNA damage is repaired over time, we conducted a repair kinetics study [Collins et al., 1997]. In this study, only one concentration per DBP was evaluated (50 μM MBA and 50 μM MCA), and different times of recovery or repair were used (0, 45, 90, 135, 180, 225, and 270 min). After treatment, each treated culture was washed with RPMI 1640 medium and resuspended in complete medium preheated to 37°C (except the negative control and time 0 samples); after that, cultures were incubated at 37°C in a 5% CO_2 incubator until its specific time of repair. After each recovery period, cell samples were resuspended in PBS and follow the same steps like in the alkaline comet assay. For the evaluation of the repair kinetics, DNA repair capacity was calculated using the average value of the Olive tail moment and from the formula: percent DNA repair capacity = [(DNA damage immediately after treatment − DNA damage at the time t of repair)/(DNA damage immediately after treatment − DNA damage of untreated cells)] × 100.

Detection of Induced Oxidative Damage

To determine the induction of oxidized bases, one concentration per each DBP was evaluated (30 μM MBA and 30 μM MCA). After cell lysis, slides were washed three times (5 min, 4°C) in a enzyme buffer solution (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8.0). Then, aliquots of 100 μL of buffer, containing the bacterial enzymes endo III or fpg (enzyme concentration 1/1,000) or no enzyme (control), were dropped onto the agarose and incubated for 30 min at 37°C. After enzyme treatments, cell samples were processed as in the standard alkaline comet assay.

Micronucleus Test

Three milliliters of a TK6 culture (500,000 cells/mL) was set up in complete medium. Aliquots of 30 μL of each DBP concentration were added to the cultures. All treatments were added at the beginning of the incubation, and cultures were kept for 48 hr at 37°C in a 5% CO_2 atmosphere. In lymphocyte cultures, 24 hr after the initiation, 50 μL of each MBA concentration was added to a 5-mL culture. Mitomycin C (MMC) and DMSO were used as positive and negative controls, respectively. For each DBP concentration and control, two replicates were made. Lymphocyte cultures lasted for 72 hr.

The CBMN test was carried out using the standard technique proposed by Fenech [1993]. Cyt-B, at a final concentration of 6 μg/mL, was added to each culture (at the beginning and 44 hr after the start, for the TK6 and lymphocyte cultures, respectively). After incubation, cells were harvested. Cultures were centrifuged at 150g for 8 min; then, the supernatant was removed and the cells were subjected to hypotonic treatment (5 mL KCl 0.075 M, 4°C 7 min), and another centrifugation was carried out. Cells were fixed with methanol/acetic acid (3:1 vol.) at least three times. In the last centrifugation, the supernatant was eliminated and the pellet was resuspended and dropped onto clean microscope slides (two drops of 20 μL each one). After drying, cells were stained with a Giemsa solution for 7 min.

All slides were coded before scoring, which was carried out by the same person using a Leitz-Leica light microscope at 1,000× magnification, under oil immersion. The criterion for scoring MN was that described by Kirsch-Volders et al. [2000]. One thousand binucleated cells were scored and classified, according to the number of MN, to calculate the induction of MN. In addition, 500 cells were scored to calculate the cytokinesis-block proliferation index (CBPI), according to the following formula: CBPI = $(M_I + 2\,M_{II} + 3(M_{III} + M_{IV})/N$, where M_I to M_{IV} represent the number of cells with one to four nuclei, respectively, and N is the number of cells scored [Surrallés et al., 1995]. The induction of MN and the calculation of CBPI were done for each replicate, and the values presented correspond to the pooled data.

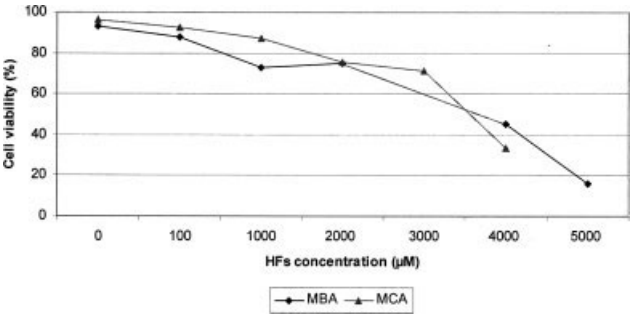

Fig. 1. Viability results after treatment of TK6 cells with MBA and MCA. Viability was measured by using the FDA/EtBr stain.

Statistical Analysis

For the comet assay, a general linear model was used to determine the statistical significance of the results. The homogeneity of variances between concentration levels was determined using Levene's test. Independent tests using Dunnett's correction for multiple test adjustment were performed to compare each level of concentration to the negative control when the overall F-test was significant. A result was considered statistically significant at $P < 0.05$. All the analyses were performed using SAS proc MIXED v9.1 (SAS Institute, Cary, NC). For the MN test, data for the binucleated cells with MN were compared for each treatment using the one-tailed Fisher's exact test. The χ^2 test was used for the analysis of CBPI among treatments.

RESULTS

According to the FDA/EtBr viability assay, both compounds (MBA and MCA) were cytotoxic in TK6 cells. The cytotoxicity of the two chemicals is similar in the lower concentrations but, at the highest one (4,000 μM), MCA appears to be slightly more cytotoxic (Fig. 1).

The results obtained in the comet assay (Fig. 2) clearly indicate the genotoxic potential of MBA. This brominated furanone induces a direct concentration-response effect, and all concentrations tested were able to produce even more damage than that produced by the positive control (H_2O_2 2,000 μM). The genotoxic potential of MCA is similar (Fig. 2), also showing a direct concentration

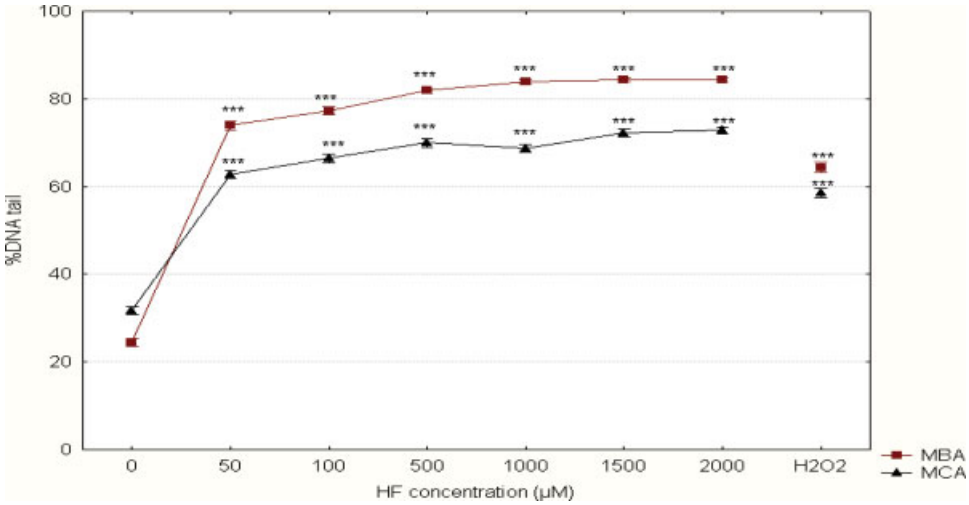

Fig. 2. Genotoxicity of MBA and mucochloric acid MCA in the comet assay after 3 hr of treatment. Genetic damage was measured as the percentage of DNA in tail. Statistical significance: $***P < 0.001$. Data represent the average of two experiments; bars, SE. H_2O_2, positive control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

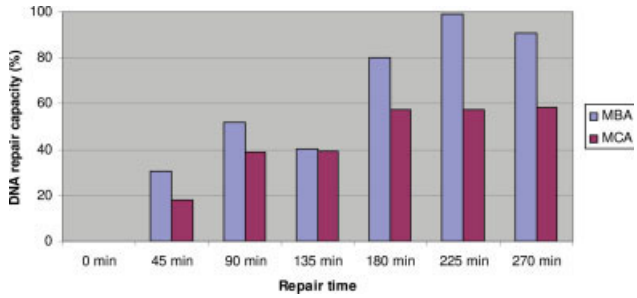

Fig. 3. DNA repair ability in TK6 cells treated with MBA (50 μM) and MCA (50 μM) for 3 hr and then maintained in fresh medium for 0, 45, 90, 135, 180, 225, and 270 min. Data represent the average of two experiments; bars, SE. H_2O_2, positive control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

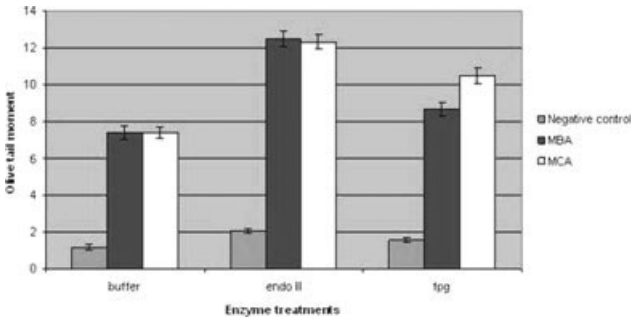

Fig. 4. Effect of the enzyme (endo III and fpg) treatments in TK6 cells previously treated with MBA (30 μM) or MCA (30 μM). Data represent the average of two experiments.

effect. We conclude that both compounds are highly genotoxic, the brominated one being more effective in the comet assay.

The repair kinetic experiments reveal that the DNA damage induced by MBA is repaired over time (Fig. 3). About 50% of the initial damage is repaired at 90 min, 80% at 180 min, and after 225 min almost all damage had been removed, showing that the cells repair the primary genetic damage caused by MBA, but not very fast. The repair kinetics and repair capacity after MCA treatment are somewhat different (see also Fig. 3); the cells being unable to remove all the damage produced by MCA. At the beginning, the curve representing the percentage of DNA repair capacity vs. the repair time shows a slight slope. About 20% of the damage had been repaired at the first 45 min and 40% is repaired between 90 and 135 min. Afterward, some further damage is repaired but only about 60% of the initial damage induced by MCA is removed. Some unrepaired damage could probably be fixed.

The levels of oxidized bases induced by MBA and MCA, which can be detected by the treatment with fpg and endo III enzymes, are shown in Figure 4. For both furanones, the damage detected with endo III treatment was higher than that detected with fpg treatment, which indicates that they induce more damage in pyrimidines than in purines. In both agents, the endo III treatment increased by 65% the level of DNA damage induced by each HF alone. Nevertheless, when both agents were challenged with fpg, a higher oxidative damage was observed with MCA (about more than 40% the level of DNA damage induced by MCA); thus, MCA was able to induce more oxidative damage than MBA.

In the comet assay, both furanones demonstrated their high ability to interact with DNA and to cause primary DNA damage. In contrast, only MCA was able to increase

TABLE I. Micronuclei (MN), Binucleated Cells with MN (BNMN), and CBPI Values Observed in TK6 Cells Treated with Mucobromic Acid (MBA) and Mucochloric Acid (MCA)

	Distribution of MN in BN cells							Distribution of cells according to the number of nuclei				
Concentration (μM)	0	1	2	3	>3	Total MN	BNMN (‰)	1	2	3	4	CBPI
MBA												
0	973	22	5	0	0	32	27	55	214	10	221	2.35
1	979	20	0	0	1[a]	25	21	40	209	23	228	2.42
5	977	22	1	0	0	24	23	47	279	16	158	2.25**
10	968	32	0	0	0	32	32	128	318	15	39	1.85***
50	Cytotoxic							Cytotoxic				
100	Cytotoxic							Cytotoxic				
MMC	913	80	6	1	0	95	87***	106	355	16	23	1.87***
MCA												
0	981	18	1	0	0	20	19	44	243	25	188	2.34
0.1	985	13	1	1	0	18	15	61	257	19	163	2.24
1	985	15	0	0	0	15	15	65	252	20	163	2.24*
5	994	6	0	0	0	6	6	88	306	5	101	2.04***
10	966	28	5	0	1[a]	43	34*	215	233	10	42	1.67***
50	Cytotoxic							Cytotoxic				
MMC (0.3 μM)	895	93	9	2	1[b]	121	105***	179	306	10	5	1.67***

Statistically significant from control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).
[a]BN cells with 5 MN.
[b]BN cells with 4 MN.

the frequency of MN. The number of MN in binucleated TK6 cells, the distribution of cells according to the number of nuclei, and the CBPI after the treatment with furanones are summarized in Table I. As observed, MBA was unable to induce MN; it only produced a decrease in the CBPI values, being completely cytotoxic at the higher concentrations assayed (50 and 100 μM).

The chlorinated furanone MCA was able to induce a significant increase in the frequency of MN, but only at the highest concentration tested (10 μM). Besides, it induced significant increases in cytotoxicity, as indicated by the CBPI values.

An additional experiment was carried out with human lymphocytes treated with MBA to confirm that the negative results obtained for MN were not due to the type of cell used. The results of this experiment are shown in Table II and confirm the inability of MBA to induce MN in mammalian cells. As expected, in all MN experiments performed, the MMC treatments (positive control) lead to significant positive increases in the MN frequency.

DISCUSSION

The use of chlorine to disinfect water for human consumption resulted in a significant decrease in the number of deaths produced by waterborne pathogen diseases. Besides producing safe drinking water, chlorine disinfection also generates DBPs and their study is considered of high priority. Currently, there is a growing interest to investigate the effect of exposure to these byproducts. Several epidemiological studies relate such exposure to an increase in the incidence of bladder cancer [Chang et al., 2007; Villanueva et al., 2007]. Considering the crucial role of mutagenic events in the carcinogenic process, it is important to determine the genotoxic activity of DBPs and to clarify the type of genetic damage produced. In this context, this study adds new information on how both DBPs tested act as genotoxicants.

In relation to the genotoxicity data available for the two HFs evaluated, the amount of information on MCA is higher than that of MBA because MCA had been quantified in drinking water and forms part of CHFs (this group is of special relevance because among its compounds can be found the mutagen X, which has great genotoxic potency).

MCA is structurally related to MX and, for this reason, it has been subjected to more evaluations. The difference between MCA and MX is in the C-4; whereas MX has a dichloromethyl group, MCA has chlorine. This change influences significantly the genotoxic and mutagenic potential, MCA being approximately four orders of magnitude less mutagenic than MX [Fekadu et al., 1994].

MCA had been evaluated alone and as a CHF mixture [Mäki-Paakkanen et al., 2004]. It is a strong mutagen in bacterial gene mutation assays, and several findings suggest that it is a SOS-dependent mutagen. Its molecular mutational spectrum is completely different from the

TABLE II. Induction of Micronuclei (MN), Binucleated Cells with MN (BNMN), and CBPI Values in Cultured Lymphocytes Treated with Mucobromic Acid (MBA)

	Distribution of MN in BN cells							Distribution of cells according to the number of nuclei				
Concentration (μM)	0	1	2	3	>3	Total MN	BNMN (‰)	1	2	3	4	CBPI
0	995	5	0	0	0	5	5	320	173	1	6	1.37
0.5	992	7	1	0	0	9	8	298	185	9	8	1.44
1	995	5	0	0	0	5	5	337	159	2	2	1.33
5	995	5	0	0	0	5	5	319	172	4	5	1.38
10	991	7	2	0	0	11	10	297	191	5	7	1.43
50	989	11	0	0	0	11	11	330	164	3	3	1.35
MMC (0.3 μM)	970	30	0	0	0	30	30***	382	116	2	0	1.24***

Statistically significant from control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

other CHFs, inducing primarily G:C → A:T transitions with a 4:1 preference of the second position of the CCC codon [Hyttinen et al., 1995; Knasmüller et al., 1996]. Fekadu et al. [1994] demonstrated that MCA is a direct-acting mutagen because the addition of a metabolic activation system (rat S-9 mix or bovine serum albumin) in DNA repair assays with *E. coli* resulted in almost a complete loss of its DNA damaging activity. MCA had also been evaluated for its aneugenic/clastogenic potential in three MN studies; one with plants and the others with mammalian cells. The first one [Helma et al., 1995] showed a dose-dependent increase of MN in pollen mother cells of *Tradescantia* when MCA was applied directly to the inflorescence, but not when applied on the stems. The other studies [Le Curiex et al., 1999; UNEP, 2003] were performed on mouse lymphoma cells, L5178Y and V79 Chinese hamster lung cells, respectively. This showed that MCA can induce a significant genotoxic effect and an increase of the frequency of MN at the highest concentration (25 μM) in L5178Y, and from 23.7 μM in V79 cells. These results are in accordance with our MN results, showing the capacity of MCA to increase the frequency of MN (at 10 μM), and would confirm the ability of this HF to induce chromosome damage.

In the other assay (comet test), MCA demonstrated a high capacity to induce DNA damage in TK6 cells. These findings agree with the results reported by Mäki-Paakkanen et al. [2001] in CHO cells. In both studies, MCA showed a dose-dependent effect, from 5 μg/mL (29.6 μM) in CHO cells, and from 50 μM in TK6 cells (the lowest concentration tested). According to these results, and from other studies that demonstrated the ability of MCA to induce sister chromatid exchanges, chromosome aberrations, and duodenal nuclear anomalies of B6C3F1 mice [Daniel et al., 1991; Mäki Paakkanen et al., 2001], it can be stated that MCA is a genotoxic agent with clastogenic activity.

With respect to the brominated hydroxyfuranone MBA, as far as we know this is the first study evaluating its genotoxicity in mammalian cells. In in vitro studies, MBA was able to form adducts with adenosine and guanosine [Kronberg et al., 1996]. MBA was also positive in the Ames test without metabolic activation, being almost twice as mutagenic as MCA [LaLonde and Leo, 1994]. This brominated DBP had been recently identified in drinking water in a U.S. National Occurrence Study [Krasner et al., 2006], especially in water with high amount of bromide. Our results from the two complementary assays performed demonstrate the high capacity of MBA to induce DNA damage, although it is unable to increase to a significant extent the frequency of MN. Therefore, MBA can be considered to have a high genotoxic potential. When comparing the results from the two HFs, the brominated one appears to be more genotoxic, in agreement with other studies that compare the effects of brominated vs. chlorinated compounds [LaLonde and Leo, 1994; Kargalioglu et al., 2002; Plewa et al., 2002, 2004; Myllykangas et al., 2003].

Our results show that an important component of the genotoxic effect produced by both HFs is the oxidative damage. In the case of MCA, this was even more relevant than the direct effect produced by the chemical itself. Both HFs can induce oxidized forms of pyrimidines and purines, the proportion of oxidized pyrimidines being far superior. The possibility of quantifying this damage, only revealed by endo III and fpg treatments, shows the high capacity of MCA and MBA to induce oxidative damage, with MCA being more effective. There is also a difference between the repair kinetics of the damage induced by both compounds. In the case of MBA, it is easily repaired but for MCA the repair is not complete, because only about 60% of the induced damage is removed and the rest is possibly fixed.

The kind of genetic damage detected in the comet assay is primary DNA damage, which can be repaired fast; but the more important damage in terms of risk is the fixed DNA damage. This damage can be adequately evaluated by means of the MN test. Only MCA was

effective in both assays and, regardless of the clearly positive results of MBA in the Comet test, it was unable to increase the frequency of MN.

We conclude that the two HFs tested, MBA and MCA, are highly effective in the comet assay, the brominated compound being the most genotoxic. As indicated by the repair kinetic studies, the damage induced by MBA is easily repaired but, in the case of MCA, only about 60% of DNA damage is repaired. In addition, both chemicals are capable of inducing high levels of oxidative damage. Finally, it can be indicated that although MBA is not effective in producing chromosome damage, as detected as MN in binucleated cells, MCA increases the MN frequency. These results are of interest when dealing with the potential risk associated with the consumption of water containing such chemicals. Nevertheless, taking into account that several HFs are able to induce point mutations in *Salmonella*, studies are required to determine whether these compounds may also produce point mutations in mammalian cells.

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