Illinois-Indiana Sea Grant Final Report

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Development of a Comparative Toxicity Database for U.S. EPA Priority Drinking Water Disinfection By-Products

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ABSTRACT

In order to generate a quantitative, direct comparison amongst classes of drinking water disinfection by-products (DBPs), we developed and calibrated *in vitro* mammalian cell cytotoxicity and genotoxicity assays to integrate the analytical biology with the analytical chemistry of these important environmental contaminants. In this study we determined the chronic cytotoxicity and acute genotoxicity of the haloacetonitriles and haloacetamide DBPs. These data demonstrate that these two emerging classes of nitrogenous DBPs follow a general rule that iodinated N-DBPs are more cytotoxic and genotoxic than their brominated and chlorinated analogues. These results are important in light of the generation of iodinated DBPs and N-DBPs that may result from the use of alternative disinfectants.

INTRODUCTION

Drinking water disinfection by-products (DBPs) are formed unintentionally when a disinfectant reacts with natural organic matter and/or bromide/iodide that are present in the raw water [1]. Although the acute benefits of drinking water disinfection are acknowledged, the health risks due to long-term DBP exposure are not well understood [2-4]. Epidemiology studies provide moderate evidence for associations of DBPs with adverse pregnancy outcomes [5], and some DBPs are

mutagens, carcinogens, teratogens, or developmental toxicants [4, 6-8]. The goal of this Illinois-Indiana Sea Grant research project was to generate an *in vitro* mammalian cell chronic cytotoxicity and acute genotoxicity database that will focus on priority drinking water disinfection by-products (DBPs) and related compounds. This research adds to a comparative database and links the analytical chemistry and analytical biology of the priority DBPs identified in the U.S. EPA Nationwide Occurrence Study [9]. Most of the priority DBPs are not commercially available, however, we have access to small quantities (<50 mg) of these agents that were synthesized for EPA for use as analytical chemical standards. The mammalian cell cytotoxicity and genotoxicity database will serve as a practical resource for the water treatment community for use in their decisions on disinfection practice. This research problem is covered by IISG topic area Water for the Future, "Develop a better understanding of the fate and effect of toxic chemicals...." The objectives of this research proposal were to, (i) select EPA priority nitrogenous DBPs, (ii) conduct a chronic mammalian cell cytotoxicity analysis on these DBPs, (iii) rank-order the DBPs based on their cytotoxicity and compare them to our current published DBP database and to positive control toxicants, (iv) determine the genomic DNA-damaging capacity of each DBP in mammalian cells, and (v) rank-order each DBP for mammalian cell genotoxic potency and compare to our current published database and to positive control carcinogens.

MATERIALS AND METHODS

Biological and Chemical Reagents

General reagents were purchased from Fisher Scientific Co. (Itasca, IL) and Sigma Chemical Co. (St. Louis, MO). Media and fetal bovine serum (FBS) were purchased from Hyclone Laboratories (Logan, UT) or from Fisher Scientific Co. (Itasca, IL).

Chinese Hamster Ovary Cells

Chinese hamster ovary (CHO) cells are widely used in toxicology. The transgenic CHO cell line AS52 [10, 11] was derived from the parental line, CHO K1-BH4 [12, 13]. Clone 11-4-8 was isolated from AS52 by Dr. E. Wagner and it expresses a stable chromosome complement, a consistent cell doubling time as well as functional p53 protein [14-16]. Stock cultures of the CHO cells were frozen in a solution of 90% fetal bovine serum (FBS):10% dimethylsulfoxide (DMSO) (v/v) and stored at −80°C. Cells were grown on glass culture plates in Hams F12 medium plus 5% FBS at 37°C in a humidified atmosphere of 5% CO_2. The cells exhibit normal morphology, express cell contact inhibition and grow as a monolayer without expression of neoplastic foci. CHO cells were transferred when the culture became confluent.

Selection and Source of DBPs and Associated Chemical Agents

For this research we focused our attention on the nitrogen-containing DBPs; specifically the halonitriles and the haloacetamides. Most of these emerging DBPs are not commercially available. However, through our collaboration with Dr. Susan Richardson, U. S. EPA, most of the priority DBPs were synthesized as analytical standards and small amounts (approximately 50 mg) were provided for this study. Reagent grade or higher chemical agents were purchased from commercial vendors when available. The DBPs and related chemicals were shipped to the laboratory, logged into a database, and usually stored in dark conditions at 4°C. Prior to the biological experiments a 1 M or a 2 M solution of the DBP was prepared in dimethylsulfoxide (DMSO).

This stock solution was immediately stored under dark conditions at −22°C. For each experiment freshly prepared serial dilutions into Hams F12 medium were made to treat the mammalian cells. A list of the sources and purities of the DBPs and related chemicals analyzed in this study is presented in Tables 1 and 2.

The haloacetonitriles (HANs) are an unregulated class of N-DBPs. From previous studies, specific HANs were toxic including bromoacetonitrile (BAN), chloroacetonitrile (CAN), dibromoacetonitrile (DBAN), dichloroacetonitrile (DCAN), trichloroacetonitrile (TCAN), and bromochloroacetonitrile (BCAN) (Table 1).

Table 1. Characteristics of the haloacetonitriles analyzed in this study

Haloacetonitrile	Abbreviation	CAS №	Chemical Formula	MW	Source and Purity
Iodoacetonitrile	IAN	624-75-9	C_2H_2NI	166.95	CanSyn/ EPA (>98%)
Bromoacetonitrile	BAN	590-17-0	C_2H_2NBr	119.95	Chem Service Lot 168-122A (97%)
Dibromoacetonitrile	DBAN	3252-43-5	C_2HNBr_2	198.84	Chem Service Lot 343-96B (97%)
Bromochloroacetonitrile	BCAN	83463-62-1	$C_2HNBrCl$	154.39	Chem Service Lot 350-1D(tech grade)
Chloroacetonitrile	CAN	107-14-2	C_2H_2NCl	75.50	Chem Service Lot 340-20A (99.5%)
Dichloroacetonitrile	DCAN	3018-12-0	C_2HNCl_2	109.94	Chem Service Lot 340-34A (99.5%)
Trichloroacetonitrile	TCAN	545-06-2	C_2NCl_3	144.39	Aldrich Chem. Co (98%)

The haloacetamides are an unregulated class of N-DBPs. In this research we analyzed the mammalian cell chronic cytotoxicity and acute genotoxicity of a series of 13 haloacetamides. A description of the haloacetamides analyzed is presented in Table 2.

Table 2. Characteristics of the haloacetamides analyzed in this study

Haloacetamide	Abbreviation	CAS No.	Chemical Formula	MW	Source and Purity
Iodoacetamide	IAcAm	144-48-9	C_2H_4INO	184.96	Sigma-Aldrich, >97 %
Diiodoacetamide	DIAcAm	5875-23-0	$C_2H_3I_2NO$	310.85	CanSyn Chem Corp., 99 %
Bromoiodoacetamide	BIAcAm	62872-36-0	C_2H_3BrINO	263.856	CanSyn Chem Corp., 85 %
Chloroiodoacetamide	CIAcAm	62872-35-9	C_2H_3ClINO	219.405	CanSyn Chem Corp., >95 %
Bromoacetamide	BAcAm	683-57-8	C_2H_4BrNO	137.96	Sigma-Aldrich, 98 %

Table 2. Characteristics of the haloacetamides analyzed in this study					
Haloacetamide	Abbreviation	CAS No.	Chemical Formula	MW	Source and Purity
Dibromoacetamide	DBAcAm	598-70-9	$C_2H_3Br_2NO$	216.86	CanSyn Chem Corp., >95 %
Tribromoacetamide	TBAcAm	594-47-8	$C_2H_2Br_3NO$	295.75	CanSyn Chem Corp., >95 %
Bromochloroacetamide	BCAcAm	62872-34-8	$C_2H_3BrClNO$	172.41	CanSyn Chem Corp., 95 %
Dibromochloroacetamide	DBCAcAm	855878-13-6	$C_2H_2Br_2ClNO$	251.305	CanSyn Chem Corp., >95 %
Bromodichloroacetamide	BDCAcAm	98137-00-9	$C_2H_2BrCl_2NO$	206.85	CanSyn Chem Corp., >95 %
Chloroacetamide	CAcAm	79-07-2	C_2H_4ClNO	93.51	Sigma-Aldrich, >95 %
Dichloroacetamide	DCAcAm	683-72-7	$C_2H_3Cl_2NO$	127.96	Sigma-Aldrich, 98 %
Trichloroacetamide	TCAcAm	594-65-0	$C_2H_2Cl_3NO$	162.40	Sigma-Aldrich, 99 %

CHO Cell Chronic Cytotoxicity Assay

This assay measures the reduction in cell density as a function of DBP concentration over a period of approximately 3 cell divisions (72 h). Chronic cytotoxicity to CHO cells was measured using a modification of an assay we developed for the analysis of DBPs [17, 18]. Flat-bottom, tissue culture 96-well microplates were employed; 8 replicate wells were prepared for each concentration of a specific DBP. Eight wells were reserved for the blank control consisting of 200 μL of F12 medium + 5% FBS. The negative control consisted of 8 wells containing 100 μL of a titered CHO cell suspension (3×10^4 cells/mL) plus 100 μL F12 + FBS. The wells for the remaining columns contained 3,000 CHO cells, F12 + FBS and a known concentration of a DBP for a total of 200 μL. To prevent cross-over contamination between wells due to volatilization of the test agent, a sheet of sterile AlumnaSeal™ (RPI Corporation, Mt. Prospect, IL) was pressed over the wells before the microplate was covered. The microplate was placed on a rocking platform for 10 min to uniformly distribute the cells and then placed in a tissue culture incubator for 72 h. After incubation, each well was gently aspirated, fixed in 100% methanol for 10 min and stained for 20 min with a 1% crystal violet solution in 50% methanol. The plate was gently washed, and 50 μL of dimethylsulfoxide (DMSO) was added to each well for 20 min. The plate was analyzed in a BioRad microplate reader at 595 nm. The data were automatically recorded and transferred to an Excel spreadsheet in a microcomputer connected to the microplate reader. The blank-corrected absorbancy value of the negative control (cells with medium only) was set at 100%. The absorbancy for each treatment group well was converted into a percentage of the negative control. For each DBP concentration, 8 replicate wells were analyzed per experiment and the experiments were repeated two to three times. Data from individual experiments were normalized

to the averaged percent of the negative control; these data were plotted as a concentration-response curve. A one-way analysis of variance test was conducted with the normalized data representing each microplate well. If a significant F value of $P \leq 0.05$ was obtained, a Holm-Sidak multiple comparison analysis was conducted.

Single Cell Gel Electrophoresis Assay

Single cell gel electrophoresis (SCGE) is a molecular genetic assay that quantitatively measures the level of genomic DNA damage induced in individual nuclei of treated cells [19, 20]. The day before treatment, 2×10^4 CHO cells were added to each microplate well in 200 μL of F12 + 5% FBS and incubated. The next day, the cells were washed with Hank's balanced salt solution (HBSS) and treated with a series of concentrations of a specific DBP in F12 medium without FBS in a total volume of 25 μL for 4 h at 37°C, 5% CO_2. The wells were covered with sterile AlumnaSeal™. After incubation, the cells were washed 2× with HBSS and harvested with 50 μL of 0.01% trypsin + 53 μM EDTA. The trypsin was inactivated with 70 μL of F12 + FBS. Acute cytotoxicity was measured from a 10 μL aliquot of cell suspension mixed with 10 μL of 0.05% trypan blue vital dye in phosphate-buffered saline (PBS). SCGE data were not used if the acute cytotoxicity exceeded 30%. The remaining cell suspension from each well was embedded in a layer of low melting point agarose prepared with PBS upon clear microscope slides that were previously coated with a layer of 1% normal melting point agarose prepared with deionized water and dried overnight. The cellular membranes were removed by an overnight immersion in lysing solution at 4°C. The slides were placed in an alkaline buffer (pH 13.5) in an electrophoresis tank, and the DNA was denatured for 20 min. The microgels were electrophoresed at 25 V, 300 mA (0.72 V/cm) for 40 min at 4°C. The microgels were neutralized with Tris buffer (pH 7.5) rinsed in cold water, dehydrated in cold methanol, dried at 50°C, and stored at room temperature in a covered slide box. For analysis, the microgels were hydrated in cold water for 30 min and stained with 65 μL of ethidium bromide (20 μg/mL) for 3 min. The microgels were rinsed in cold water and analyzed with a Zeiss fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. For each experiment, 2 microgels were prepared per treatment group. Randomly chosen nuclei (25 per microgel) were analyzed using a charged coupled device camera. A computerized image analysis system (Komet version 3.1, Kinetic Imaging Ltd., Liverpool, UK) was employed to determine the SCGE tail moment value (integrated value of migrated DNA density multiplied by the migration distance) of the nuclei as indices of DNA damage. The digitalized data were automatically transferred to a computer based spreadsheet for subsequent statistical analysis. Within each experiment, a negative control, a positive control (3.8 mM ethylmethanesulfonate) and 9 concentrations of a specific DBP were analyzed concurrently. The experiments were repeated a minimum of 3 times for each DBP. SCGE tail moment values are not normally distributed, thus the median tail moment value for each microgel was determined and averaged. Averaged median values express a normal distribution [21] and were used with a one-way analysis of variance test with the microgel used as the unit of measure. If a significant F value of $P \leq 0.05$ was obtained, a Holm-Sidak multiple comparison analysis was conducted with a power of 0.8 at $\alpha = 0.05$.

RESULTS AND DISCUSSION

CHO Cell Cytotoxicity Analysis of the Haloacetonitriles

The CHO cell chronic cytotoxicity of the seven haloacetonitriles analyzed in this study are presented in Table 3. In the table, the lowest concentration of a specific haloacetonitrile was identified by the ANOVA test statistic that induced a significant toxic response as compared to their concurrent negative controls. The %C½ value was the concentration of the haloacetonitrile that induced a 50% reduction of the cell density as compared to the negative controls. Finally, the R^2 refers to the fit of the regression analysis from which the %C½ value was calculated. All concentrations are presented in molar (M) units of measure. The lowest concentrations that induced a significant cytotoxic response ranged from 100 nM (iodoacetonitrile) to 50 μM (chloroacetonitrile) (Table 3). The %C½ values ranged from 2.8 μM (dibromoacetonitrile) to 160 μM (trichloroacetonitrile). The rank order for cytotoxicity (highest to lowest) of the 7 haloacetonitriles based on their %C½ values was dibromoacetonitrile > bromoacetonitrile > iodoacetonitrile > bromochloroacetonitrile > dichloroacetonitrile > chloroacetonitrile > trichloroacetonitrile.

Table 3. CHO cell chronic cytotoxicity of the haloacetonitrile DBPs

Compound	Lowest Tox. Conc. (M)	R^2	%C½ (M)	ANOVA Test Statistic
Bromoacetonitrile	1.0×10^{-6}	0.98	3.21×10^{-6}	$F_{11,228} = 98.3$; $P \leq 0.001$
Bromochloroacetonitrile	7.0×10^{-6}	0.96	8.46×10^{-6}	$F_{11,171} = 36.2$; $P \leq 0.001$
Chloroacetonitrile	5.0×10^{-5}	0.99	6.83×10^{-5}	$F_{13,188} = 65.9$; $P \leq 0.001$
Dibromoacetonitrile	1.0×10^{-6}	0.99	2.85×10^{-6}	$F_{11,179} = 271.5$; $P \leq 0.001$
Dichloroacetonitrile	1.0×10^{-5}	0.99	5.73×10^{-5}	$F_{10,171} = 63.4$; $P \leq 0.001$
Iodoacetonitrile	1.0×10^{-7}	0.98	3.30×10^{-6}	$F_{12,163} = 148.4$; $P \leq 0.001$
Trichloroacetonitrile	2.5×10^{-5}	0.93	1.60×10^{-4}	$F_{17,282} = 36.8$; $P \leq 0.001$

A comparison of the relative cytotoxicity of the haloacetonitriles analyzed in this study is presented in Figure 1.

CHO Cell Genotoxicity Analysis of the Haloacetonitriles

In this study seven haloacetonitriles were analyzed for their ability to induce genomic DNA damage in CHO cells; the results are presented in Table 4. The lowest concentration of a specific haloacetonitrile was identified by the ANOVA test statistic that induced significant genomic DNA strand breakage (as measured by SCGE median tail moment values) as compared to their concurrent negative controls. The SCGE genotoxic potency value was calculated for each chemical from the concentration-response curve. It represents the midpoint of the curve within the concentration range that expressed above 70% cell viability of the treated cells. Finally, the R^2 refers to the fit of the regression analysis from which the SCGE genotoxicity value was calculated. All concentrations are presented in molar (M) units of measure.

A comparison of the CHO cell genotoxicity for the haloacetonitriles is presented in Figure 2. The SCGE genotoxic potency values ranged from 29.7 μM (dibromoacetonitrile) to 2.75 mM (dichloroacetonitrile). The descending rank order for genotoxicity based on their SCGE genotoxicity potency values was dibromoacetonitrile > iodoacetonitrile ≈ bromoacetonitrile > bromochloroacetonitrile > trichloroacetonitrile > dichloroacetonitrile.

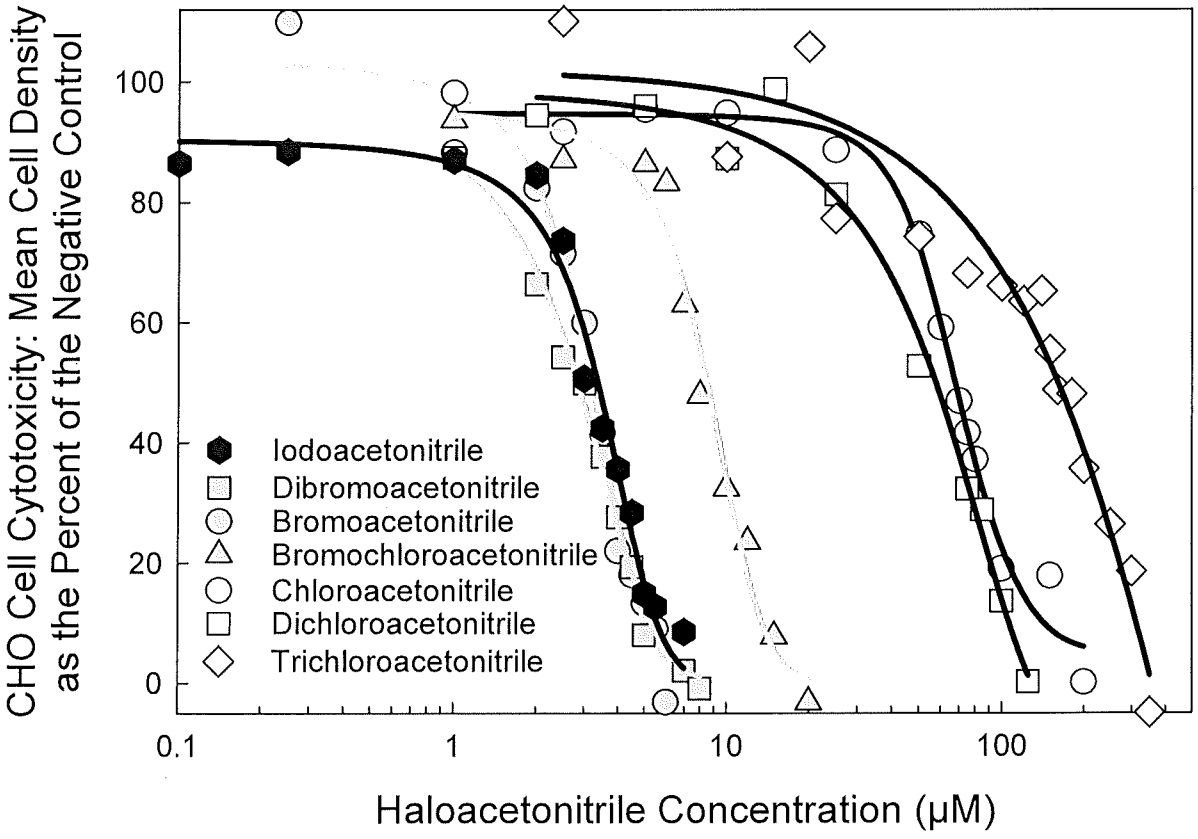

Figure 1 Comparison of the concentration-response curves for the CHO cell chronic cytotoxicity of seven haloacetonitriles.

Table 4. CHO cell genotoxicity of the haloacetonitrile DBPs

Compound	Lowest Genotox. Conc. (M)	R^2	SCGE Gen. Potency (M)	ANOVA Test Statistic
Bromoacetonitrile	4.00×10^{-5}	0.99	3.85×10^{-5}	$F_{6,36} = 32.7$; $P \leq 0.001$
Bromochloroacetonitrile	2.50×10^{-4}	0.98	3.24×10^{-4}	$F_{10,41} = 19.1$; $P \leq 0.001$
Chloroacetonitrile	2.50×10^{-4}	0.99	6.01×10^{-4}	$F_{11,42} = 28.9$; $P \leq 0.001$
Dibromoacetonitrile	3.00×10^{-5}	0.95	2.97×10^{-5}	$F_{9,46} = 46.1$; $P \leq 0.001$
Dichloroacetonitrile	2.40×10^{-3}	0.98	2.75×10^{-3}	$F_{17,62} = 14.2$; $P \leq 0.001$
Iodoacetonitrile	3.00×10^{-5}	0.98	3.71×10^{-5}	$F_{10,53} = 46.6$; $P \leq 0.001$
Trichloroacetonitrile	1.00×10^{-3}	0.98	1.01×10^{-3}	$F_{7,32} = 30.5$; $P \leq 0.001$

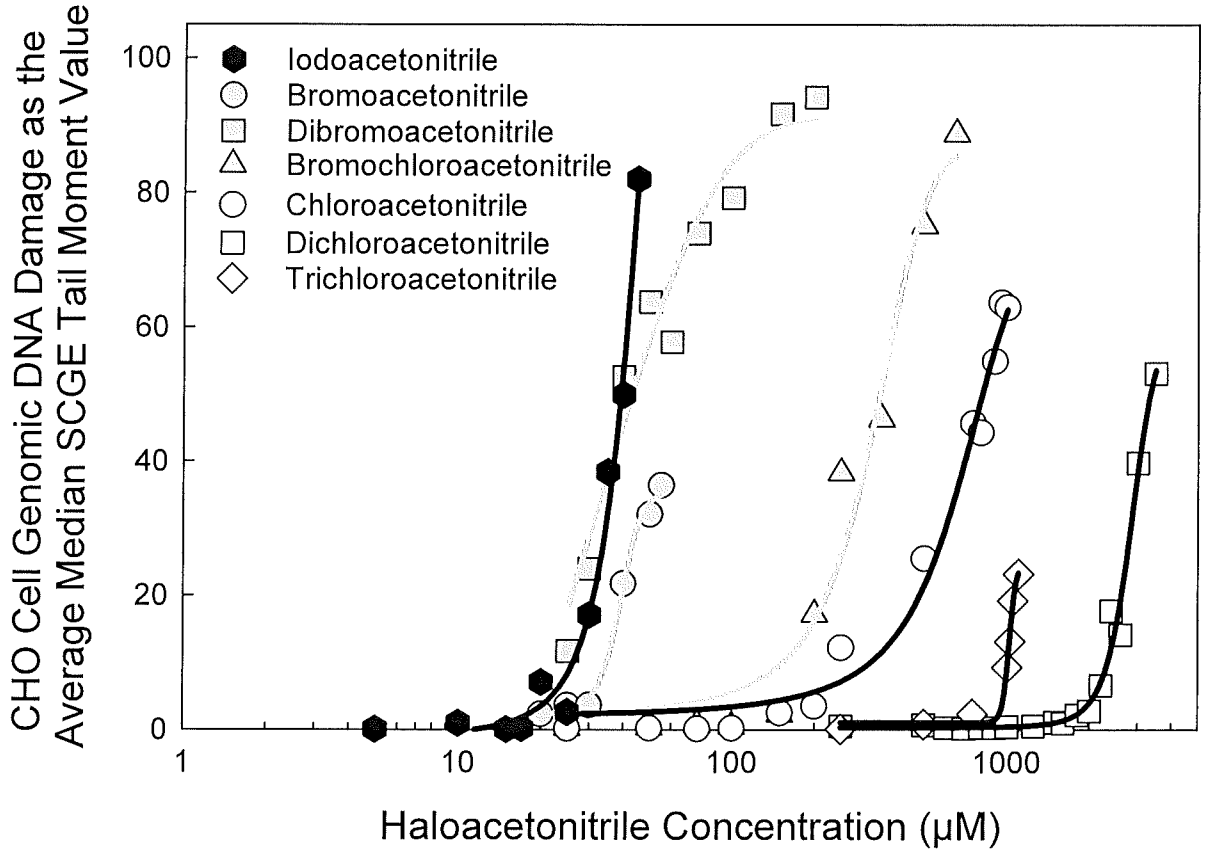

Figure 2 Comparison of the SCGE genotoxicity concentration-response curves of seven haloacetonitriles.

Haloacetamides

Thirteen haloacetamides were evaluated in this study, bromoacetamide, bromochloroacetamide, bromodichloroacetamide, bromoiodoacetamide, chloroacetamide, chloroiodoacetamide, dibromoacetamide, dibromochloroacetamide, dichloroacetamide, diiodoacetamide, iodoacetamide, tribromoacetamide, and trichloroacetamide (Table 2).

CHO Cell Cytotoxicity Analysis of the Haloacetamides

The CHO cell chronic cytotoxicity of the 13 haloacetamides analyzed in this study are presented in Table 5. In the table, the lowest concentration of a specific haloacetamide was identified by the ANOVA test statistic that induced a significant toxic response as compared to their concurrent negative controls. The %C½ value was the concentration of the haloacetamide that induced a 50% reduction of the cell density as compared to the negative controls. Finally, the R^2

refers to the fit of the regression analysis from which the %C½ value was calculated. All concentrations are presented in molar (M) units of measure. The lowest concentration that induced a significant cytotoxic response ranged from 25 nM (diiodoacetamide) to 800 μM (dichloroacetamide) (Table 5). The %C½ values ranged from 678 nM (diiodoacetamide) to 2.05 mM (trichloroacetamide). The rank order for cytotoxicity (highest to lowest) of the 13 haloacetamides based on their %C½ values was diiodoacetamide > iodoacetamide > bromoacetamide > tribromoacetamide > bromoiodoacetamide > dibromochloroacetamide > chloroiodoacetamide > bromodichloroacetamide > dibromoacetamide > bromochloroacetamide > chloroacetamide > dichloroacetamide > trichloroacetamide.

A comparison of the relative cytotoxicity of the haloacetamides analyzed in this study is presented in Figure 3.

Table 5. CHO cell chronic cytotoxicity of the haloacetamide DBPs

Compound	Lowest Tox. Conc. (M)	R^2	%C½ (M)	ANOVA Test Statistic
Bromoacetamide	5.00×10^{-7}	0.99	1.89×10^{-6}	$F_{12, 282} = 57.15$; $P \leq 0.001$
Bromochloroacetamide	1.00×10^{-6}	0.96	1.71×10^{-5}	$F_{16, 183} = 111.05$; $P \leq 0.001$
Bromodichloroacetamide	2.00×10^{-6}	0.98	8.68×10^{-6}	$F_{10, 197} = 173.96$; $P \leq 0.001$
Bromoiodoacetamide	2.00×10^{-6}	0.98	3.81×10^{-6} [a]	$F_{10, 164} = 85.99$; $P \leq 0.001$
Chloroacetamide	7.50×10^{-5}	0.98	1.48×10^{-4}	$F_{13, 176} = 99.20$; $P \leq 0.001$
Chloroiodoacetamide	2.00×10^{-6}	0.96	5.97×10^{-6}	$F_{14, 193} = 111.78$; $P \leq 0.001$
Dibromoacetamide	2.50×10^{-6}	0.99	1.22×10^{-5}	$F_{11, 283} = 174.56$; $P \leq 0.001$
Dibromochloroacetamide	1.00×10^{-6}	0.96	4.75×10^{-6}	$F_{9, 174} = 40.56$; $P \leq 0.001$
Dichloroacetamide	8.00×10^{-4}	0.95	1.92×10^{-3}	$F_{12, 271} = 79.20$; $P \leq 0.001$
Diiodoacetamide	2.50×10^{-8}	0.98	6.78×10^{-7}	$F_{10, 149} = 144.35$; $P \leq 0.001$
Iodoacetamide	5.00×10^{-7}	0.98	1.42×10^{-6}	$F_{17, 332} = 133.23$; $P \leq 0.001$
Tribromoacetamide	2.00×10^{-6}	0.97	3.14×10^{-6}	$F_{10, 275} = 122.62$; $P \leq 0.001$
Trichloroacetamide	5.00×10^{-4}	0.96	2.05×10^{-3}	$F_{11, 251} = 77.05$; $P \leq 0.001$

[a] The calculated %C½ value for bromoiodoacetamide alone assuming an additive model for the diiodoacetamide and dibromoacetamide contaminants was 3.35×10^{-6} M.

CHO Cell Genotoxicity Analysis of the Haloacetamides

In this study 13 haloacetamides were analyzed for their ability to induce genomic DNA damage in CHO cells; the results are presented in Table 6. In the table, the lowest concentration of a specific haloacetamide was identified by the ANOVA test statistic that induced significant genomic DNA strand breakage (as measured by SCGE median tail moment values) as compared to their concurrent negative controls. The SCGE genotoxic potency value was calculated for each chemical from the concentration-response curve. It represents the midpoint of the curve within the concentration range that expressed above 70% cell viability of the treated cells. Finally, the R^2 refers to the fit of the regression analysis from which the SCGE genotoxicity value was calculated. All concentrations are presented in molar (M) units of measure. A comparison of the CHO cell genotoxicity for these agents is presented in Figure 4.

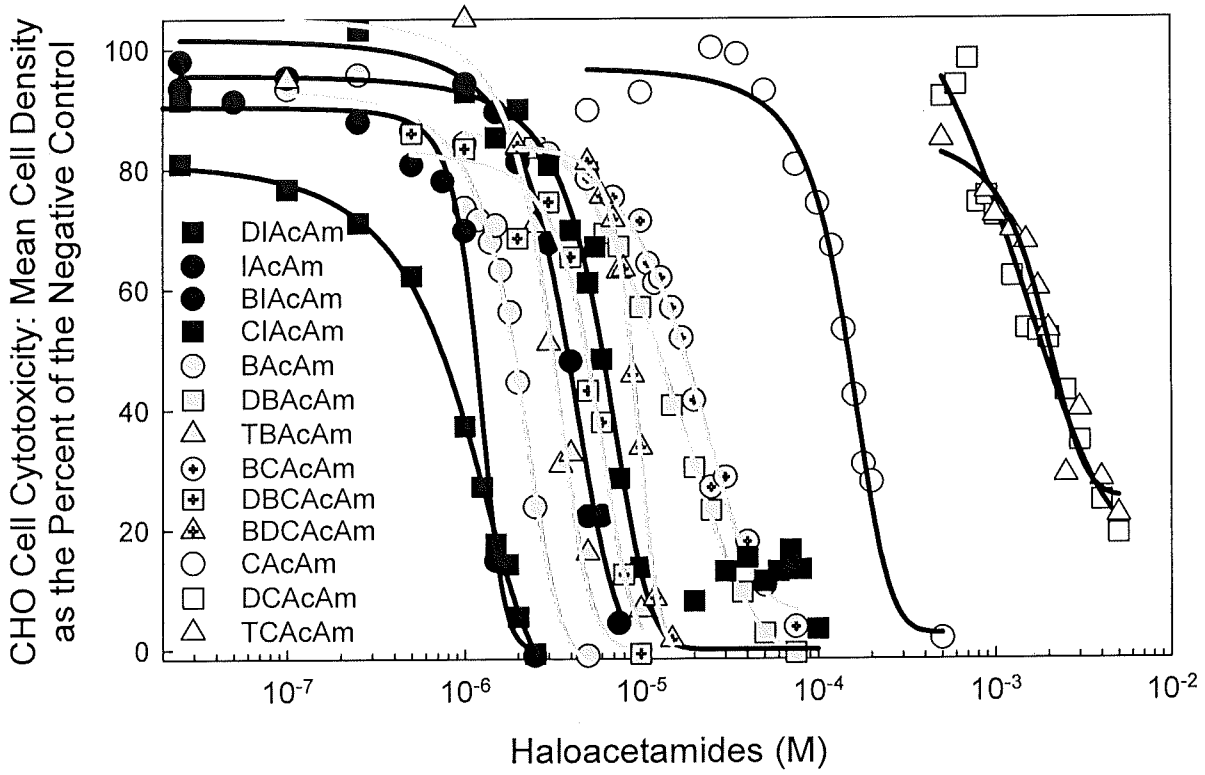

Figure 3 Comparison of the CHO cell chronic cytotoxicity concentration-response curves for the haloacetamides analyzed in this study. The abbreviations are DIAcAm = diiodoacetamide, IAcAm = iodoacetamide, BIAcAm = bromoiodoacetamide, CIAcAm = chloroiodoacetamide, BAcAm = bromoacetamide, DBAcAm = dibromoacetamide, TBAcAm = tribromoacetamide, BCAcAm = bromochloroacetamide, DBCAcAm = dibromochloroacetamide, BDCAcAm = bromodichloroacetamide, CAcAm = chloroacetamide, DCAcAm = dichloroacetamide, and TCAcAm = trichloroacetamide

The SCGE genotoxic potency values ranged from 32.5 μM (tribromoacetamide) to 6.5 mM (trichloroacetamide). The descending rank order for genotoxicity based on their SGE genotoxic potency value was tribromoacetamide > diiodoacetamide ≈ iodoacetamide > bromoacetamide > dibromoacetamide > bromoiodoacetamide > bromodichloroacetamide > chloroiodoacetamide > bromochloroacetamide > dibromoacetamide > chloroacetamide, with dichloroacetamide not genotoxic.

Table 6. CHO cell genotoxicity of the haloacetamide DBPs and related chemicals

Compound	Lowest Genotox. Conc. (M)	R^2	SCGE Gen. Potency (M)	ANOVA Test Statistic
Bromoacetamide	2.50×10^{-5}	0.99	3.68×10^{-5}	$F_{9,38} = 29.77$; $P \leq 0.001$
Bromochloroacetamide	4.00×10^{-4}	0.99	5.83×10^{-4}	$F_{9,48} = 53.86$; $P \leq 0.001$
Bromodichloroacetamide	7.50×10^{-5}	0.99	1.46×10^{-4}	$F_{9,39} = 58.41$; $P \leq 0.001$

Bromoiodoacetamide	2.50×10^{-5}	0.99	7.21×10^{-5} [a]	$F_{10, 54} = 29.38$; $P \leq 0.001$
Chloroacetamide	7.50×10^{-4}	0.99	1.38×10^{-3}	$F_{11, 46} = 25.02$; $P \leq 0.001$
Chloroiodoacetamide	2.00×10^{-4}	0.99	3.02×10^{-4}	$F_{17, 62} = 35.19$; $P \leq 0.001$
Dibromoacetamide	5.00×10^{-4}	0.99	7.44×10^{-4}	$F_{10, 47} = 21.09$; $P \leq 0.001$
Dibromochloroacetamide	2.50×10^{-5}	0.98	6.94×10^{-5}	$F_{8, 37} = 185.59$; $P \leq 0.001$
Dichloroacetamide	NA	NA	NS $>1\times10^{-2}$	$F_{11, 34} = 1.026$; $P = 0.417$
Diiodoacetamide	2.50×10^{-5}	0.98	3.39×10^{-5}	$F_{11, 60} = 29.12$; $P \leq 0.001$
Iodoacetamide	3.00×10^{-5}	0.99	3.41×10^{-5}	$F_{15, 43} = 13.11$; $P \leq 0.001$
Tribromoacetamide	3.00×10^{-5}	0.97	3.25×10^{-5}	$F_{17, 62} = 35.19$; $P \leq 0.001$
Trichloroacetamide	5.00×10^{-3}	0.98	6.54×10^{-3}	$F_{9, 50} = 5.75$; $P \leq 0.001$

NS = not statistically different from the negative control, NA = non applicable. [a] The calculated SCGE genotoxic potency value for bromoiodoacetamide alone assuming an additive model for the diiodoacetamide and dibromoacetamide contaminants was 1.62×10^{-5} M.

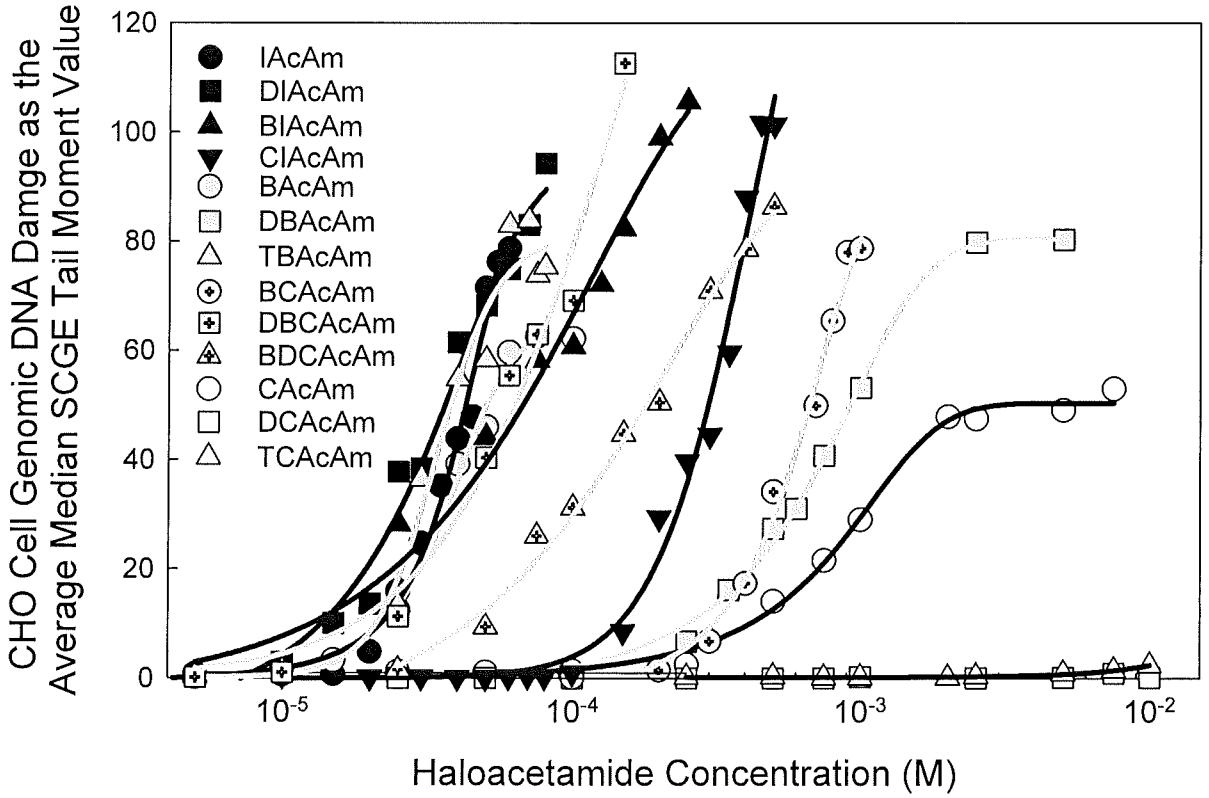

Figure 4 Comparison of the SCGE genotoxicity concentration-response curves of 13 haloacetamides. The abbreviations are IAcAm = iodoacetamide, DIAcAm = diiodoacetamide, BIAcAm = bromoiodoacetamide, CIAcAm = chloroiodoacetamide, BAcAm = bromoacetamide, DBAcAm = dibromoacetamide, TBAcAm = tribromoacetamide, BCAcAm = bromochloroacetamide, DBCAcAm = dibromochloroacetamide, BDCAcAm = bromodichloroacetamide, CAcAm = chloroacetamide, DCAcAm = dichloroacetamide, and TCAcAm = trichloroacetamide

CONCLUSIONS

The conclusions from this study include the following two items:

1. Nitrogen-containing DBPs and related agents, including haloacetonitriles and haloacetamides were far more cytotoxic and genotoxic than DBPs that did not contain nitrogen (haloacids, halomethanes) that were analyzed in previous studies.
2. These results are very relevant during the evaluation period for the U.S. EPA Stage 2 D/DBP Rule [22]. Emerging DBPs, especially iodinated and nitrogen-containing agents, are more cytotoxic and generally induce a greater level of genomic DNA damage in mammalian cells than currently regulated DBPs.

POTENTIAL APPLICATIONS OR BENEFITS

1. *In vitro* mammalian cell methodologies offer rapid, precise and sensitive means to evaluate DBPs or concentrated complex mixtures from drinking water. These methods should be part of the toxicological analysis of finished drinking water as one measure of water quality.
2. This research demonstrates that wide ranges of cytotoxic and genotoxic responses are expressed within and among DBP chemical classes. One class of DBPs cannot serve as a surrogate to predict the occurrence or toxicity of other DBPs or classes of DBPs.
3. Those emerging DBPs identified as U.S. EPA priority compounds that were analyzed in this study are more cytotoxic and genotoxic than currently regulated DBPs.
4. Drinking water utilities should conduct a detailed chemical and *in vitro* toxicological analysis of their finished waters throughout the distribution system when altering disinfection methods.
5. Although not currently regulated, special attention should be given to the generation of iodinated DBPs. Generally these agents are more cytotoxic and genotoxic than their brominated and chlorinated analogues.
6. In general nitrogen-containing DBPs are substantially more cytotoxic and genotoxic in mammalian cells than similar DBPs that do not contain nitrogen (e.g. halonitromethanes versus halomethanes). These agents, and the water treatment plant conditions, source water conditions and disinfectant protocols that lead to the generation of nitrogen-containing DBPs should be controlled and monitored.

KEYWORDS

CHO cells, drinking water disinfection byproducts, chlorination, chloramination, comet assay

LAY SUMMARY

The drinking water community provides an exceedingly important public health service by its generation of high quality, safe and palatable tap water. The disinfection of drinking water in public facilities primarily employs chemical disinfectants such as chlorine, chloramines, ozone

and chlorine dioxide. These disinfectants are oxidants that convert naturally occurring and synthetic organic material, bromide, and iodide in the raw water into chemical disinfection by-products (DBPs). DBPs are an unintended consequence and were first discovered over 30 years ago. Each disinfection method generates a different spectrum and distribution of DBPs; to date over 600 DBPs have been identified. While reducing the public health risk to acute infection by waterborne pathogens, the unintended generation of DBPs poses a chronic health risk. DBPs represent an important class of environmentally hazardous chemicals that are regulated by the U.S. Environmental Protection Agency (U.S. EPA) and carry long-term human health implications. Epidemiological studies demonstrated that individuals who consume chlorinated drinking water have an elevated risk of cancer. DBPs have been linked to reproductive and developmental effects, including the induction of spontaneous abortions in humans. Mammalian cell cytotoxicity and genotoxicity data provided a rank ordering of the relational toxicities of regulated and emerging DBPs and related agents both within an individual chemical class and among classes. Alternative disinfectants generate new DBP compounds and alter the distribution of DBP chemical classes. The water supply community will be able to consider these factors when employing alternatives to chlorine disinfection. In addition these data will be available to prioritize DBPs for future *in vivo* toxicological studies and risk assessment.

PUBLICATIONS

Muellner, M. G.; Wagner, E. D.; McCalla, K.; Richardson, S. D.; Woo, Y. T.; Plewa, M. J., Haloacetonitriles vs. regulated haloacetic acids: Are nitrogen containing DBPs more toxic? *Environ. Sci. Technol.* **2007**, *41*, (2), 645-651.

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Undergraduate/Graduate Names and Degree

Kristin McCalla, undergraduate research student, B.S. 2007
Mark Muellner, graduate student, Ph.D. 2008

Related Projects

M.J. Plewa and E.D. Wagner, Quantitative comparative mammalian cell cytotoxicity and genotoxicity of selected classes of drinking water disinfection by-products, American Water Works Association Research Foundation, $150,000, 2004-2007.

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