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Mammalian Cell Cytotoxicity and Genotoxicity of Disinfection By-Products

Subject Area: Water Quality




Mammalian Cell Cytotoxicity and Genotoxicity of Disinfection By-Products

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The Foundation's mission is to advance the science of water to improve the quality of life. To achieve this mission, the Foundation sponsors studies on all aspects of drinking water, including resources, treatment, distribution, and health effects. Funding for research is provided primarily by subscription payments from close to 1,000 water utilities, consulting firms, and manufacturers in North America and abroad. Additional funding comes from collaborative partnerships with other national and international organizations and the U.S. federal government, allowing for resources to be leveraged, expertise to be shared, and broad-based knowledge to be developed and disseminated.

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Mammalian Cell Cytotoxicity and Genotoxicity of Disinfection By-Products

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FOREWORD

The Water Research Foundation (Foundation) is a nonprofit corporation that is dedicated to the implementation of a research effort to help utilities respond to regulatory requirements and traditional high-priority concerns of the industry. The research agenda is developed through a process of consultation with subscribers and drinking water professionals. Under the umbrella of a Strategic Research Plan, the Research Advisory Council prioritizes the suggested projects based upon current and future needs, applicability, and past work; the recommendations are forwarded to the Board of Trustees for final selection. The Foundation also sponsors research projects through the unsolicited proposal process; the Collaborative Research, Research Applications, and Tailored Collaboration programs; and various joint research efforts with organizations such as the U.S. Environmental Protection Agency, the U.S. Bureau of Reclamation, and the Association of California Water Agencies.

This publication is a result of one of these sponsored studies, and it is hoped that its findings will be applied in communities throughout the world. The following report serves not only as a means of communicating the results of the water industry's centralized research program but also as a tool to enlist the further support of the nonmember utilities and individuals.

Projects are managed closely from their inception to the final report by the Foundation's staff and large cadre of volunteers who willingly contribute their time and expertise. The Foundation serves a planning and management function and awards contracts to other institutions such as water utilities, universities, and engineering firms. The funding for this research effort comes primarily from the Subscription Program, through which water utilities subscribe to the research program and make an annual payment proportionate to the volume of water they deliver and consultants and manufacturers subscribe based on their annual billings. The program offers a cost-effective and fair method for funding research in the public interest.

A broad spectrum of water supply issues is addressed by the Foundation's research agenda: resources, treatment and operations, distribution and storage, water quality and analysis, toxicology, economics, and management. The ultimate purpose of the coordinated effort is to assist water suppliers to provide the highest possible quality of water economically and reliably. The true benefits are realized when the results are implemented at the utility level. The Foundation's trustees are pleased to offer this publication as a contribution toward that end.

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Water Research Foundation

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Finally, of the greatest value to any university research project is the group of undergraduate and graduate students who contribute to the success of the work. The following students deserve special attention: Mark G. Muellner, Kang-Mei Hsu, and Kristin McCalla.

EXECUTIVE 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 byproducts (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.

Although chlorine has been used for over 100 years as a water disinfectant, the majority of DBPs present in drinking water have yet to be chemically characterized. Many drinking water treatment plants use multiple chemical disinfection methods. With only approximately 30% (median value) of the total organic halide (TOX) identified to specific DBP chemical classes, and a small fraction of these evaluated for their biological and toxicological effects, it is clear that a great deal of work remains in the characterization of DBPs.

This project represents basic research funded by the Water Research Foundation (Foundation) under its unsolicited proposal program. The work to generate quantitative, comparative *in vitro* mammalian cell toxicity data of emerging DBPs and related compounds is the first systematic cytotoxicity and genotoxicity analysis of its kind. This study represents the third branch of a tripartite foundation leading toward the analysis and better understanding of the occurrence and impact of emerging DBPs. Two prior pioneering studies, under the auspices of the U.S. EPA, established the need for a quantitative, comparative analysis of the cytotoxicity and genotoxicity of emerging DBPs. The U.S. EPA reported a mechanism-based structure-activity relationships (SAR) analysis for the carcinogenic potential ranking of DBPs. A list of priority DBPs was generated based on this ranking that met the criteria of, (i) having a good probability of being detected in actual drinking water samples, (ii) having insufficient cancer bioassay data for risk assessment, and, (iii) having structural features/alerts or short-term predictive assays indicative of carcinogenic potential. The priority DBPs included, iodinated trihalomethanes and other halomethanes, haloacids, haloacetonitriles, haloketones, halonitromethanes, haloaldehydes, halogenated furanones, haloacetamides, and nonhalogenated carbonyls. The U.S. EPA Nationwide Occurrence Study included in its work this list of more than 50 priority DBPs as well as currently regulated DBPs. This landmark study, completed in 2002, generated quantitative occurrence information for new and emerging DBPs for prioritizing future health effects studies. In the present work reported here, the *in vitro* mammalian cell toxicological database was built upon the data from the U.S. EPA SAR study and the U.S. EPA Nationwide Occurrence Study. 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.

RESEARCH OBJECTIVES

This study represents the largest quantitative and comparative analysis of *in vitro* mammalian cell cytotoxicity and genotoxicity of DBPs from the priority DBPs and the U.S. EPA Nationwide Occurrence Study. These empirical data will aid in the further development of mechanism-based SAR techniques. Identification of the most toxic DBPs to mammalian cells will serve the drinking water utility community in their goal of constantly improving the quality and delivery of safe drinking water. The specific objectives of this project are listed below.

1. Select specific priority DBPs from the list generated by the U.S. EPA and with the advice from the Foudnation's project advisory committee.
2. Conduct a concentration-response analysis for each DBP to determine its chronic cytotoxicity in mammalian cells (72 hour exposure).
3. Rank-order the DBPs based on their cytotoxicity and generate a comparative database.
4. Determine the genomic DNA-damaging capacity of each DBP in mammalian cells using quantitative molecular biological analysis.
5. Rank-order each DBP for its mammalian cell genotoxicity and develop a comparative database.
6. Using the toxicity data generated from this study, conduct SAR analysis of the DBPs.

APPROACH

Priority DBPs and Related Compounds

Reagent grade or higher chemical agents were purchased from commercial vendors. Many of the emerging priority DBPs and related compounds were not commercially available. Through a collaboration with Dr. Susan Richardson (National Exposure Research Laboratory, U.S. Environmental Protection Agency, Athens, Georgia) most of the priority DBPs were synthesized as analytical standards.

Chinese Hamster Ovary Cells

Chinese hamster ovary (CHO) cells, line AS52, clone 11-4-8 were used in this study. This cell line (and its parental line K1-BH4) has been widely used for *in vitro* toxicology research; AS52 was used in previous DBP toxicity studies. The CHO cells were maintained in Ham's F12 medium containing 5% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2.

Analytical Methods

The analytical methods primarily employed two quantitative biological assays. The CHO cell chronic cytotoxicity assay measures the reduction in cell density as a function of DBP concentration over a period of approximately 3 cell divisions. For each DBP approximately ten con-

centrations were evaluated in a range that expressed no observed toxic response to complete cell killing. For each DBP concentration eight independent replicate cell cultures were analyzed per experiment and each experiment was repeated two to four times. 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. CHO cells were exposed to a series of DBP concentrations for 4 h, the cells were harvested, the nuclei were fixed into microgels and electrophoresed under alkaline conditions. For each experiment, 2 microgels were prepared per treatment group. The nuclei were analyzed using a charged coupled device camera. A computerized image analysis system was employed to determine the SCGE tail moment value (integrated value of migrated DNA density multiplied by the migration distance) of the nuclei as a measure of DNA damage. The digitalized data were automatically transferred to a computer based spreadsheet for subsequent statistical analysis. The experiments were repeated a minimum of 3 times for each DBP.

Data Handling and Safety

Experimental designs for each DBP analyzed were prepared and recorded in data books. The information for each experiment, identified by a unique experiment number, was placed in a file within an Excel spreadsheet. The raw data and the statistical and graphical information were kept in computer files.

Safety is a principal concern in the laboratory. Manipulations of toxic, genotoxic and/or carcinogenic chemicals were conducted using disposable papers and gloves in a certified stage two containment biological/chemical safety hood. The disposal of hazardous material was in compliance with the University of Illinois regulations. All staff involved on this project received safety training under the auspices of the University of Illinois Institutional Biological Safety Committee. The regulations of the Division of Research Safety of the University of Illinois were implemented throughout this project.

CONCLUSIONS

1. Forty-seven compounds from six DBP chemical classes that included halomethanes, haloacetic acids, >2C-haloacids, haloacetonitriles, haloacetamides and haloacetaldehydes were systematically analyzed for their *in vitro* chronic cytotoxicity and acute genotoxicity in a mammalian cell assay. Of the 47 compounds analyzed, only 4 are currently regulated by the U.S. EPA. In addition to studying the regulated trihalomethanes, the 5 regulated haloacetic acids were analyzed in previous work and are part of the overall database.
2. The CHO cell cytotoxicity and genotoxicity results, when added to other published data constituted the largest comparative database of 66 DBPs and related agents.
3. The CHO cell chronic cytotoxicity of the DBPs encompassed concentrations over 5 log orders of magnitude with diiodoacetamide the most cytotoxic agent and bromodichloromethane the least cytotoxic.
4. The CHO cell genotoxicity of the DBPs encompassed concentrations over 5 orders of magnitude. A majority (75.8%) induced significant levels of genomic DNA damage. In this group iodoacetic acid was the most genotoxic.

5. For CHO cell cytotoxicity, the rank order from most toxic to least toxic for the DBP classes was haloacetaldehydes > haloacetamides > halonitromethanes > haloacetonitriles > >2C-haloacids > haloacetic acids > halomethanes.
6. For induced genomic DNA damage in CHO cells, the rank order from the most genotoxic to the least genotoxic of the DBP classes was haloacetonitriles > haloacetamides > halonitromethanes > haloacetaldehydes > haloacetic acids > >2C-haloacids > halomethanes (trihalomethanes only).
7. Although there was an overall significant correlation between chronic cytotoxicity and acute genotoxicity, this correlation did not apply to all DBP chemical classes.
8. Within an individual chemical class, there was a correlation between chronic cytotoxicity and acute genotoxicity for the haloacetamides, haloacetaldehydes, and to a lesser degree the halonitromethanes. The haloacetic acids, >2C-haloacids and the haloacetonitriles showed no such correlation.
9. In a balanced comparison of iodinated, brominated and chlorinated DBP analogues, the cytotoxicity and genotoxicity of the iodinated DBPs was greater than that of their brominated or chlorinated analogues with the chlorinated analogues the least toxic.
10. Nitrogen-containing DBPs and related agents, including haloacetonitriles, haloacetamides, and halonitromethanes were far more cytotoxic and genotoxic than DBPs that did not contain nitrogen (haloacids, halomethanes). However, as a class the haloacetaldehydes were very cytotoxic and genotoxic and are DBPs of concern.
11. These results are very relevant during the evaluation period for the U.S. EPA Stage 2 D/DBP Rule. 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.

RECOMMENDATIONS

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.

FUTURE RESEACH

This research, when combined with other published studies, generated the first and largest systematic *in vitro* mammalian cell databases on chronic cytotoxicity and genotoxicity of DBPs. These databases allow for the direct comparison of the biological impact of DBPs and related compounds within and among DBP chemical classes. The utility of these results allows for identifying those DBPs or related compounds that are of highest concern for adverse effects upon the public health and the environment. The CHO cell chronic cytotoxicity and acute SCGE genotoxicity database should continue to expand to include novel DBPs as they are discovered by analytical chemists. High priority DBPs such as the brominated and possibly iodinated halofuranones, novel nitrogen-containing DBPs, higher molecular weight DBPs, and especially iodinated analogues, should be evaluated and added to the CHO cell cytotoxicity and genotoxicity database.

It was beyond the scope of this research project to integrate the occurrence and concentration of DBPs with the toxicological data. An important future goal would be to develop a methodology that integrates the occurrence, concentration, mammalian cell cytotoxicity and genotoxicity of DBPs and to compare the predicted effect on finished water generated with different disinfection methods.

Finally, toxicogenomics analysis — the study of the complex interaction between the cellular genome, chemicals in the environment, and disease — must be applied to DBP research. Toxicogenomics is the new frontier in toxicology. Future work should involve toxicogenomic research on regulated and emerging DBPs using non-transformed human cells (non-cancer cell lines). Focus should be on those pathways that impact DNA damage and repair as well as metabolism, especially where known human polymorphisms exist. This approach may lead to new human biomarkers associated with enhanced sensitivity to DBPs. Finally work should be conducted to determine if specific human subpopulations have enhanced sensitivity to risks associated with DBPs including cancer, birth defects or reproductive failure.

CHAPTER 1
INTRODUCTION

BACKGROUND

One can easily argue that the drinking water community provides an exceedingly important public health service for the nation by its generation of high quality, safe and palatable tap water. Each day approximately 250,000 public water purification facilities in the United States provide over 1.3×10^{10} liters of high quality, drinking water to 90% of the population (Richardson et al. 2007). The disinfection of drinking water in public facilities primarily uses chemical disinfectants such as chlorine, chloramines, ozone and chlorine dioxide (Minear and Amy 1996). These disinfectants are also oxidants that convert natural organic material (NOM), 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 (Rook 1974; Bellar, Lichtenbert, and Kroner 1974). Each disinfection method generates a different spectrum and distribution of DBPs (Zhang et al. 2000; Hua and Reckhow 2007); to date over 600 DBPs have been identified (Richardson 1998). While reducing the public health risk of acute infection by waterborne pathogens, the generation of DBPs poses a chronic health risk. Many DBPs have been identified as toxic agents, genotoxins and carcinogens (Umemura and Kurokawa 2006; National Cancer Institute 1976; National Toxicology Program 1978; Bull et al. 1990; Bull et al. 1995; Moudgal, Lipscomb, and Bruce 2000; Moore and Chen 2006; Melnick et al. 2007; Richardson et al. 2007). DBPs represent an important class of environmentally hazardous chemicals that carry long-term human health implications (Richardson et al. 2007; Gopal et al. 2007; World Health Organization 2006; Simmons et al. 2004; Boorman et al. 1999; Ohanian, Mullin, and Orme 1989). Epidemiological studies have defined an association between individuals who consume chlorinated drinking water have an elevated risk of cancer of the bladder, stomach, pancreas, kidney, and rectum as well as Hodgkin's and non-Hodgkin's lymphoma (Bove, Rogerson, and Vena 2007; Michaud et al. 2007; Villanueva et al. 2007; Cantor et al. 2006; Villanueva et al. 2006; McDonald and Komulainen 2005; IARC 2004; Komulainen 2004; Villanueva et al. 2004; Cantor 1997; Morris et al. 1992; Koivusalo et al. 1994). Certain DBPs have been associated with adverse reproductive and developmental effects (Andrews et al. 2004; Ward, Rogers, and Hunter 2000; Boorman et al. 1999; Richard and Hunter 1996; Hunter et al. 1996; Hunter and Tugman 1995), including the induction of spontaneous abortions in humans, adverse pregnancy outcomes or birth difects (Swan et al. 1998; Waller et al. 1998; Bove, Shim, and Zeitz 2002; Dodds and King 2001; Nieuwenhuijsen et al. 2000; Magnus et al. 1999; Bove et al. 1995; Aschengrau, Zierler, and Cohen 1993; Savitz et al. 2005).

Although chlorine has been used for over 100 years as a water disinfectant, the majority of DBPs present in disinfected water have yet to be chemically defined (Zhang et al. 2000; Krasner et al. 2006; Weinberg et al. 2002; Weinberg 1999; Hua and Reckhow 2007). Identified DBPs ranged from approximately 30 to 60 % of the total organic halide (TOX) produced in chlorinated drinking water (Stevens et al. 1990). Many drinking water treatment plants use multiple chemical disinfection methods. A summary of the percentages of DBP classes of the water treatment facilities surveyed in the U.S. EPA Nationwide Occurrence Study is presented in Figure 1.1 (Krasner et al. 2006). With only approximately 30% of the median total organic halide identified

to specific DBP chemical classes, it is clear that a great deal of work remains in the characterization of DBPs (Richardson, Simmons, and Rice 2002).

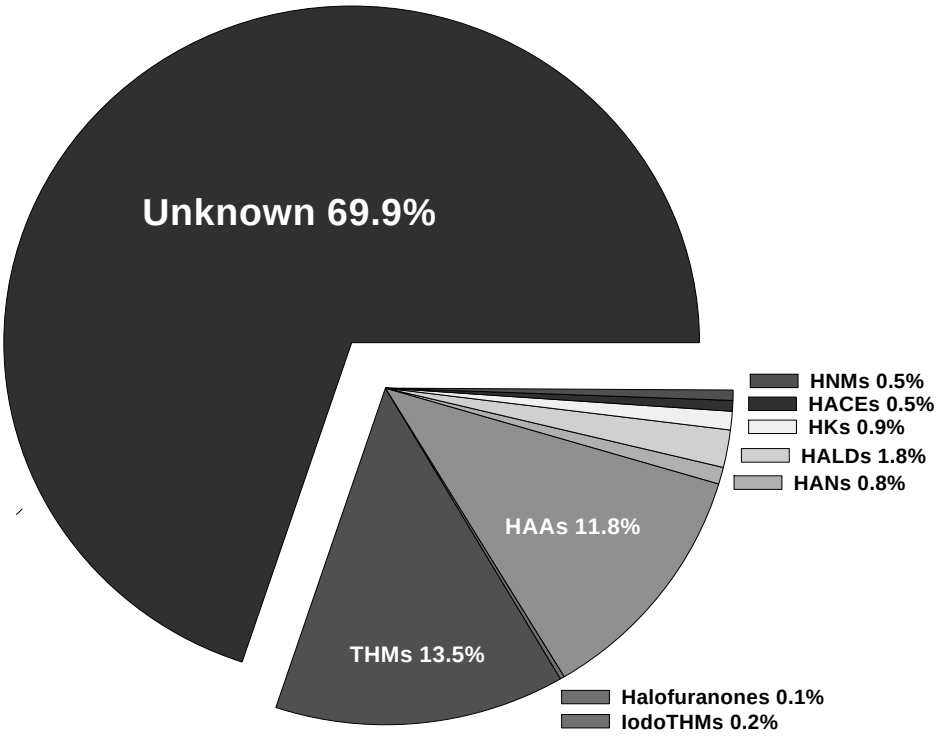


Figure 1.1 Summary of the distribution of the median values of the DBP chemical classes in water analyzed in the U.S. EPA Nationwide Occurrence Study as a component of TOX (Krasner et al. 2006). The abbreviations are THMs = trihalomethanes, HAAs = haloacetic acids, HNMs = halonitromethanes, HACEs = haloacetamides, HKs = haloketones, HALDs = haloacetaldehydes, HANs = haloacetonitriles.

Project Genesis

This project represents the third part of a tripartite foundation leading toward the analysis and better understanding of the impact of emerging DBPs on the environment (Monarca et al. 2005; Monarca et al. 2003) and the public health (Richardson et al. 2007). Two primary publications established the need for a quantitative, comparative analysis of the cytotoxicity and genotoxicity of emerging DBPs. Woo and his colleagues at the U.S. EPA reported a mechanism-based structure-activity relationships analysis for the carcinogenic potential ranking of DBPs (Woo et al. 2002). They generated a list of priority DBPs based on this ranking that met the criteria of, (i) having a good possibility of being present in drinking water, (ii) having insufficient cancer bioassay data for risk assessment, and, (iii) having structural features/alerts or short-term predictive assays indicative of carcinogenic potential. The priority DBPs identified by the research team included iodinated trihalomethanes and other halomethanes, haloacids, haloacetonitriles, haloketones, halonitromethanes, haloacetaldehydes, halogenated furanones, haloacetamides, and nonhalogenated carbonyls. The U.S. EPA Nationwide Occurrence Study (Weinberg et

al. 2002) included this list of more than 50 priority DBPs as well as currently regulated DBPs. This landmark study generated quantitative occurrence information for new and emerging DBPs for prioritizing future health effects studies (Krasner et al. 2006). The final piece in the analysis of these priority DBPs and newly identified DBPs was the present study on the *in vitro* mammalian cell cytotoxicity and genotoxicity.

Evolving U.S. EPA Regulations

In 1979 the U.S. EPA issued a regulation to control total trihalomethanes (chloroform, bromodichloromethane, dibromochloromethane, and bromoform) at an annual average of 100 μg/L (ppb) in drinking water (U. S. Environmental Protection Agency 1979). In 1998 the U. S. EPA issued the Stage 1 Disinfectants (D)/DBP Rule, which lowered permissible levels of total THMs to 80 μg/L and regulated five haloacetic acids (chloro-, dichloro-, trichloro-, bromo-, and dibromoacetic acids) at 60 μg/L, bromate at 10 μg/L, and chlorite at 1.0 mg/L (U. S. Environmental Protection Agency 1998). Stage 1 regulations required monitoring based on running annual averages in a utility's distribution system. Recently the Stage 2 D/DBP Rule was enacted that maintained the Stage 1 Rule maximum contaminant levels (MCLs) for the DBPs plus the additional restriction that the MCLs for the trihalomethanes and haloacetic acids are based on locational running annual averages (U. S. Environmental Protection Agency 2006). The Stage 2 D/DBP Rule maintains the MCLs for bromate and chlorite; however, the U.S. EPA plans to review the bromate MCL as part of their 6-year review process. Besides the United States, the United Nations World Health Organization has issued guidelines for DBPs (World Health Organization 2006) as well as the European Union (European Union 1998).

With stricter regulations for trihalomethanes and haloacetic acids, many drinking water utilities are considering changing their disinfection practices. Often, the primary disinfectant is changed from chlorine to alternative disinfectants, including ozone, chlorine dioxide, and chloramines. In some cases, chlorine is used as a secondary disinfectant following primary treatment with an alternative disinfectant. However, new issues and problems can result with changes in disinfection practice.

Distribution of DBPs as a Function of Disinfection Method

Alternative chemical disinfectants other than chlorine can substantially change the distribution of the DBP chemical classes present in the finished water (Stevens et al. 1989; Glaze and Weinberg 1993; Andrews and Ferguson 1996). It is important to note that alternative disinfectants form much less TOX than chlorine with a comparative rank order of $TOX_{chlorine} > TOX_{chloramines} > TOX_{chlorine\ dioxide} > TOX_{ozone}$. In a detailed laboratory study (Zhang et al. 2000) Suwannee river fulvic acid was added to deionized, distilled water to generate a source water of 3.0 mg/L total organic carbon (TOC). Four different disinfectants were used to treat this source water: the resulting DBPs were characterized and compared. The percentage of TOX that was accounted for by the known DBPs varied with the disinfectant. In the water treated with chlorine, the known DBPs accounted for nearly 50% of TOX. However, only 28%, 17%, and 8.3% of TOX could be represented by the known DBPs for chlorine dioxide, chloramine, and ozone, respectively. The contribution of major chemical classes of DBPs also varied with the disinfectant. These data indicate that unknown and potentially hazardous novel DBPs may be generated by using alterna-

tive disinfection methods especially when heightened levels of bromine or iodine are present in the raw waters (Richardson et al. 2006; Plewa, Wagner, Richardson et al. 2004).

OBJECTIVES

This study represents the largest quantitative and comparative analysis of *in vitro* mammalian cell cytotoxicity and genotoxicity of DBPs (and related compounds) from the priority DBPs (Woo et al. 2002) and the U.S. EPA Nationwide Occurrence Study (Weinberg et al. 2002; Krasner et al. 2006). These empirical data will aid in the further development of mechanism-based structure activity relationship (SAR) techniques. Identification of the most potent toxic DBPs to mammalian cells will serve the drinking water utility community in their goal of constantly improving the quality and delivery of safe drinking water. The specific objectives of this project are listed below.

1. Select specific priority DBPs and related compounds for use in the mammalian cell bioassays. This selection was conducted in concert with our colleagues at the U. S. EPA and with the Foundation's project advisory committee.
2. Conduct a concentration-response analysis for each DBP to determine its chronic cytotoxicity in mammalian cells (72 hour exposure).
3. Rank-order the DBPs based on their cytotoxicity and generate a comparative database.
4. Determine the genomic DNA-damaging capacity of each DBP in mammalian cells using quantitative molecular biological analysis.
5. Rank-order each DBP for its mammalian cell genotoxicity and develop a comparative database.
6. Using the toxicity data generated from this study, conduct a structure activity relationship analysis of the DBPs.

The results of this research generated the data to integrate the U.S. EPA SAR study (Woo et al. 2002) and the U.S. EPA Nationwide Occurrence Study (Krasner et al. 2006) with an *in vitro* mammalian cell toxicological database. The use of alternative disinfectants other than chlorine generates new DBP compounds and alters the distribution of DBP chemical classes. Mammalian cell cytotoxicity and genotoxicity data provided a rank ordering of the relational toxicities of the priority as well as novel DBPs and related agents both within an individual chemical class and between classes so that 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 and their related compounds for future *in vivo* toxicological studies and risk assessment.

CHAPTER 2
MATERIALS AND METHODS

INTRODUCTION

This research involved the selection of priority DBPs in various chemical classes and their toxicological analysis using sensitive, *in vitro* mammalian cell assays for chronic cytotoxicity and acute genotoxicity.

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 (Tindall et al. 1984; Tindall and Stankowski 1989) was derived from the parental K1-BH4 line (Hsie et al. 1975, 1975). Clone 11-4-8 was isolated from AS52 by Dr. E. Wagner and it expresses a stable chromosome complement and a consistent cell doubling time as well as functional p53 protein (Wagner et al. 1998, 1998; Tzang et al. 1999).

SELECTION AND SOURCE OF DBPs AND ASSOCIATED CHEMICAL AGENTS

A major problem for the toxicological analysis of emerging DBPs is that the large majority of the agents listed by Woo et al. (Woo et al. 2002) and Krasner et al. (Krasner et al. 2006) are not commercially available. However, through a collaboration with Dr. Susan Richardson, U. S. EPA, most of the priority DBPs were synthesized as analytical standards and small amounts (~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 Table 2.1.

BIOLOGICAL ASSAYS

Maintenance of CHO Cells

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.

Table 2.1.
Sources and purities of the DBPs and related chemical agents used in the *in vitro* CHO cell toxicity assays

DBP or Chemical Agent	Chemical Class	MW	Purity %	Source
Bromochloroiodomethane	Halomethane	255.58	>95	Helix Biotech.
Bromodichloromethane	Halomethane	163.83	>98	Aldrich Chem. Co.
Chlorodibromomethane	Halomethane	208.29	98	Aldrich Chem. Co.
Dibromoiodomethane	Halomethane	299.73	>95	Helix Biotech.
Tribromomethane (bromoform)	Halomethane	252.75	99	Fisher Scientific
Trichloromethane (chloroform)	Halomethane	119.38	>99	Aldrich Chem. Co.
Triiodomethane (iodoform)	Halomethane	393.73	99	Sigma-Aldrich Co.
Bromochloroacetic acid	Haloacetic acid	173.40	99	U.S. EPA
Bromodichloroacetic acid	Haloacetic acid	207.84	98	Radian Internatl.
Bromoiodoacetic acid	Haloacetic acid	264.84	>90	U.S. EPA
Chlorodibromoacetic acid	Haloacetic acid	252.29	98	Cerilliant Co.
Diiodoacetic acid	Haloacetic acid	311.84	>95	CanSyn Chem. Co.
3-Bromo-3-iodopropenoic acid	Haloacid	320.73	>99	U.S. EPA
2,3-Dibromopropenoic acid	Haloacid	229.85	>99	U.S. EPA
3,3-Dibromopropenoic acid	Haloacid	229.85	>99	U.S. EPA
2-Iodo-3-bromopropenoic acid	Haloacid	276.85	>90	U.S. EPA
2,3,3-Tribromopropenoic acid	Haloacid	308.74	95	U.S. EPA
3-Bromo-3-chloro-4-oxopentanoic acid	Haloacid	229.46	>85	CanSyn Chem. Co.
3,3-Dibromo-4-oxopentanoic acid	Haloacid	273.91	>99	U.S. EPA
2-Bromobutenedioic acid	Haloacid	194.97	>99	U.S. EPA
(*E*)-2-Iodo-3-methylbutenedioic acid	Haloacid	255.99	>95	CanSyn Chem. Co.
trans-2-Bromo-3-methylbutenedioic acid	Haloacid	208.99	>95	CanSyn Chem. Co.
Bromoacetonitrile	Halonitrile	119.95	97	Chem. Service
Bromochloroacetonitrile	Halonitrile	154.39	>95	Chem. Service
Chloroacetonitrile	Halonitrile	75.50	>99	Chem. Service
Dibromoacetonitrile	Halonitrile	198.84	97	Chem. Service
Dichloroacetonitrile	Halonitrile	109.94	>99	Chem. Service
Iodoacetonitrile	Halonitrile	166.95	98	CanSyn Chem. Co.
Trichloroacetonitrile	Halonitrile	144.39	98	Aldrich Chem. Co.
Bromoacetamide	Haloamide	137.96	98	U.S. EPA
Bromochloroacetamide	Haloamide	172.41	>99	CanSyn Chem. Co.
Bromodichloroacetamide	Haloamide	206.85	>95	CanSyn Chem. Co.
Bromoiodoacetamide	Haloamide	263.86	85	CanSyn Chem. Co.

(continued)

Table 2.1 (Continued)
Sources and purities of the DBPs and related chemical agents used in the *in vitro* CHO cell toxicity assays

DBP or Chemical Agent	Chemical Class	MW	Purity %	Source
Chloroacetamide	Haloamide	93.51	>95	U.S. EPA
Chloroiodoacetamide	Haloamide	219.41	>95	CanSyn Chem. Co.
Dibromoacetamide	Haloamide	216.86	>95	CanSyn Chem. Co.
Dibromochloroacetamide	Haloamide	251.31	>95	CanSyn Chem. Co.
Dichloroacetamide	Haloamide	127.96	98	U.S. EPA
Diiodoacetamide	Haloamide	310.85	>99	CanSyn Chem. Co.
Iodoacetamide	Haloamide	184.96	>97	Sigma Chem. Co.
Tribromoacetamide	Haloamide	295.75	>95	CanSyn Chem. Co.
Trichloroacetamide	Haloamide	162.40	99	U.S. EPA
Chloroacetaldehyde	Haloaldehyde	78.50	>95	CanSyn Chem. Co.
Dibromoacetaldehyde	Haloaldehyde	201.85	>95	CanSyn Chem. Co.
Dichloroacetaldehyde	Haloaldehyde	112.94	>95	CanSyn Chem. Co.
Tribromoacetaldehyde	Haloaldehyde	280.74	97	CanSyn Chem. Co.
Trichloroacetaldehyde	Haloaldehyde	165.40	>95	CanSyn Chem. Co.

CHO Cell Chronic Cytotoxicity Assay

The CHO cell microplate chronic cytotoxicity assay measures the reduction in cell density as a function of the concentration of the test agent over a 72 h period (Plewa et al. 2002, 2000). A 96-well flat-bottomed microplate was used to evaluate a series of chemical concentrations. One column of eight microplate wells served as the blank control consisting of 200 µL of F12 +FBS medium only. The concurrent negative control column consisted of eight wells with 3×10^3 CHO cells plus F12 +FBS medium. The wells of the remaining columns contained 3×10^3 CHO cells, F12 +FBS and a known DBP concentration in a total of 200 µL (Figure 2.1). The wells were covered with a sheet of sterile AlumnaSeal™ and the cells were incubated for 72 h at 37°C at 5% CO_2. After the treatment time, the medium from each well was aspirated, the cells fixed in methanol for 10 min and stained for 10 min with a 1% crystal violet solution in 50% methanol. The microplate was washed, 50 µL of DMSO/methanol (3:1 v/v) were added to each well, and the plate was incubated at room temperature for 10 min. The microplate was analyzed at 595 nm with a BioRad microplate reader; the absorbancy of each well was recorded and stored on a spreadsheet file (Table 2.2). This assay was calibrated and there is a direct relationship between the absorbancy of the crystal violet dye associated with the cell and the number of viable cells (Figure 2.2) (Plewa et al. 2002). A flow diagram for the CHO cell chronic cytotoxicity assay is presented in Figure 2.3.

Normalization of CHO Cytotoxicity Data and Statistical Analysis

The averaged absorbancy of the blank wells (Figure 2.1, column 2) (Table 2.2) was subtracted from the absorbancy data from each well (Table 2.3). The mean blank-corrected absorbancy value of the negative control was set at 100%. The absorbancy for each treatment group

well was converted into a percentage of the negative control (Table 2.4). This procedure normalized the data, maintained the variance and allowed the combination of data from multiple microplates (Table 2.5). For each DBP concentration, 8 replicate wells were analyzed per experiment, and the experiments were repeated 2-4×. These data were used to generate a concentration-response curve for each DBP (Figure 2.4). Regression analysis was applied to each DBP concentration-response curve, which was used to calculate the %C½ value, which is analogous to a LC_{50} value. The %C½ value is the calculated DBP concentration that induced a cell density that was 50% of the negative control (Figure 2.4).

Table 2.2
Absorbancy data from a single microplate measuring the CHO cell cytotoxicity of dibromoacetamide and the average absorbancy for the blank column

0	Blank	2.5	5	6.25	7.5	10	15	20	25	37.5	50	µM
0.333	0.095	0.264	0.269	0.251	0.216	0.230	0.173	0.154	0.125	0.111	0.106	
0.279	0.093	0.300	0.280	0.265	0.251	0.206	0.164	0.156	0.128	0.106	0.097	
0.280	0.098	0.280	0.256	0.254	0.255	0.206	0.149	0.132	0.114	0.113	0.097	
0.287	0.100	0.281	0.270	0.279	0.269	0.257	0.154	0.166	0.137	0.104	0.112	
0.322	0.100	0.297	0.277	0.250	0.239	0.226	0.152	0.153	0.139	0.110	0.108	
0.320	0.098	0.301	0.264	0.259	0.252	0.233	0.185	0.172	0.156	0.136	0.098	
0.330	0.116	0.290	0.266	0.287	0.271	0.224	0.197	0.157	0.151	0.130	0.121	
0.320	0.106	0.303	0.251	0.247	0.258	0.226	0.192	0.180	0.154	0.114	0.110	
	0.101											

Table 2.3
Blank-corrected absorbancy data and the average value for the negative control from a single microplate measuring the CHO cell cytotoxicity of dibromoacetamide

0	Blank	2.5	5	6.25	7.5	10	15	20	25	37.5	50	µM
0.232		0.163	0.168	0.150	0.115	0.129	0.072	0.053	0.024	0.010	0.005	
0.178		0.199	0.179	0.164	0.150	0.105	0.063	0.055	0.027	0.005	-0.004	
0.179		0.179	0.155	0.153	0.154	0.105	0.048	0.031	0.013	0.012	-0.004	
0.186		0.180	0.169	0.178	0.168	0.156	0.053	0.065	0.036	0.003	0.011	
0.221		0.196	0.176	0.149	0.138	0.125	0.051	0.052	0.038	0.009	0.007	
0.219		0.200	0.163	0.158	0.151	0.132	0.084	0.071	0.055	0.035	-0.003	
0.229		0.189	0.165	0.186	0.170	0.123	0.096	0.056	0.050	0.029	0.020	
0.219		0.202	0.150	0.146	0.157	0.125	0.091	0.079	0.053	0.013	0.009	
0.208												

The data from the cytotoxicity experiments were transferred to Excel spreadsheets and analyzed using the statistical and graphical functions of SigmaPlot 8.02, SigmaStat 3.1 and Table Curve 4.03 (Systat Software Inc., San Jose, CA). The crystal violet absorbancy data collected by the Bio-Rad microplate reader was saved as a text file (.txt) with the experiment number and transferred to an Excel spreadsheet. The original absorbancy data, the blank-corrected and the conversion to the percent of the negative control values were saved on the spreadsheet for each DBP analyzed. For each DBP, a summary page was prepared and all of the statistical analysis was conducted on the percent of the negative control values. A cytotoxicity concentration-response curve for each DBP was generated from the summary page and a one-way analysis of variance (ANOVA) test was conducted. If a significant F value of $P \leq 0.05$ was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted. The power of

the test statistic (1−β) was maintained as ≥0.8 at α = 0.05. An example of the statistical analysis of dibromoacetamide is presented in Table 2.6.

Table 2.4
Data normalized as the percent of the negative control from a single microplate measuring the CHO cell cytotoxicity of dibromoacetamide

0	Blank	2.5	5	6.25	7.5	10	15	20	25	37.5	50	µM
111.54		78.37	80.77	72.12	55.29	62.02	34.62	25.48	11.54	4.81	2.40	
85.58		95.67	86.06	78.85	72.12	50.48	30.29	26.44	12.98	2.40	-1.92	
86.06		86.06	74.52	73.56	74.04	50.48	23.08	14.90	6.25	5.77	-1.92	
89.42		86.54	81.25	85.58	80.77	75.00	25.48	31.25	17.31	1.44	5.29	
106.25		94.23	84.62	71.63	66.35	60.10	24.52	25.00	18.27	4.33	3.37	
105.29		96.15	78.37	75.96	72.60	63.46	40.38	34.13	26.44	16.83	-1.44	
110.10		90.87	79.33	89.42	81.73	59.13	46.15	26.92	24.04	13.94	9.62	
105.29		97.12	72.12	70.19	75.48	60.10	43.75	37.98	25.48	6.25	4.33	
8		8	8	8	8	8	8	8	8	8	8	number
99.94		90.63	79.63	77.16	72.30	60.10	33.53	27.76	17.79	6.97	2.46	Average
3.88		2.31	1.66	2.47	2.98	2.75	3.21	2.46	2.57	1.94	1.45	SE
10.98		6.54	4.70	7.00	8.44	7.77	9.08	6.95	7.26	5.49	4.09	SD

Table 2.5
Summary of normalized data as the percent of the negative control from 5 experiments measuring the CHO cell cytotoxicity of dibromoacetamide

0	2.5	5	6.25	7.5	10	15	20	25	37.5	50	75	µM
93.0	91.9	94.5										
87.3	82.8	99.3										
89.9	89.0	91.6										
101.3	94.9	91.6										
102.5	104.0	71.1										
89.9	91.9	95.2										
128.5	91.6	96.7										
106.3	116.1	137.7										
79.1		102.3	77.9	63.6	60.5	54.1	37.4	24.1	15.6	1.5	0.0	
93.8		36.2	80.5	69.7	77.7	55.9	23.3	25.1	12.6	5.4	-1.5	
87.5		74.4	69.2	60.5	59.2	45.6	37.7	21.5	9.7	3.6	-2.8	
93.8		91.3	73.8	65.6	64.1	58.2	38.5	30.8	14.1	7.9	-1.5	
94.1		92.3	81.0	87.7	68.2	54.4	42.1	28.7	30.3	6.9	0.8	
95.6		65.9	66.2	74.9	55.1	39.7	45.6	33.8	10.5	5.4	-1.8	
116.5		87.9	93.6	83.6	74.9	55.1	52.1	43.1	16.7	11.3	2.1	
138.1		82.3	66.2	83.6	29.7	69.0	3.8	39.0	-9.7		0.5	
104.4	75.38	61.15	69.23	62.31	65.38	34.62	35.38	26.15	16.15	5.00		
106.9	51.15	55.38	46.15	48.46	47.31	31.92	23.85	15.77	6.54	2.31		
105.9	52.31	70.38	44.23	41.92	44.23	13.85	20.38	15.77	0.38	-3.46		
95.4	64.23	55.77	55.38	42.69	40.38	29.23	19.23	12.31	4.23	-5.77		
93.3	66.92	54.23	50.38	60.77	46.54	24.62	24.62	19.23	9.62	0.77		
104.1	56.54	56.92	56.15	47.69	45.00	36.54	29.23	21.15	7.31	-2.31		
99.2	78.46	59.23	58.85	66.92	56.15	46.54	33.46	31.92	15.77	1.92		
90.8	72.69	83.85	56.92	71.92	53.46	60.00	38.85	25.77	13.46	1.92		
91.15	78.37	80.77	72.12	55.29	62.02	34.62	25.48	11.54	4.81	2.40		
81.54	95.67	86.06	78.85	72.12	50.48	30.29	26.44	12.98	2.40	-1.92		
85.77	86.06	74.52	73.56	74.04	50.48	23.08	14.90	6.25	5.77	-1.92		
88.46	86.54	81.25	85.58	80.77	75.00	25.48	31.25	17.31	1.44	5.29		
107.31	94.23	84.62	71.63	66.35	60.10	24.52	25.00	18.27	4.33	3.37		
107.31	96.15	78.37	75.96	72.60	63.46	40.38	34.13	26.44	16.83	-1.44		
120.77	90.87	79.33	89.42	81.73	59.13	46.15	26.92	24.04	13.94	9.62		
117.69	97.12	72.12	70.19	75.48	60.10	43.75	37.98	25.48	6.25	4.33		
111.54												
85.58												
86.06												
89.42												
106.25												
105.29												
110.10												
105.29												
40	24	32	24	24	24	24	24	24	24	23	8	Num
99.9	83.5	79.5	69.3	67.1	57.0	40.7	30.3	23.2	9.5	2.7	-0.5	Mean
2.03	3.34	3.36	2.68	2.64	2.35	2.88	2.15	1.80	1.60	0.89	0.58	SE
12.85	16.36	19.01	13.12	12.93	11.53	14.10	10.55	8.84	7.82	4.26	1.63	SD

Table 2.6
Statistical analysis of the CHO cell cytotoxicity of dibromoacetamide

Data source: Data 21 in AWWARF3089 Master 071705.SNB

Group Name	N	Missing	Mean	Std Dev	SEM
DBAcAm 0 µM	40	0	99.920	12.849	2.032
DBAcAm 2.5 µM	24	0	83.540	16.364	3.340
DBAcAm 5 µM	32	0	79.505	19.010	3.360
DBAcAm 6.25 µM	24	0	69.295	13.117	2.677
DBAcAm 7.5 µM	24	0	67.095	12.934	2.640
DBAcAm 10 µM	24	0	57.030	11.529	2.353
DBAcAm 15 µM	24	0	40.735	14.103	2.879
DBAcAm 20 µM	24	0	30.318	10.552	2.154
DBAcAm 25 µM	24	0	23.189	8.836	1.804
DBAcAm 37.5 µM	24	0	9.541	7.823	1.597
DBAcAm 50 µM	24	0	2.702	4.255	0.887
DBAcAm 75 µM	8	0	-0.545	1.630	0.576

Source of Variation	DF	SS	MS	F	P
Between Groups	11	308159.080	28014.462	174.560	<0.001
Residual	283	45417.450	160.486		
Total	294	353576.531			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	Unadjusted P	Critical Level
DBAcAm 0 µM vs. DBAcAm 50 µM	97.218	29.326	0.000	0.005
DBAcAm 0 µM vs. DBAcAm 37.5 µM	90.379	27.631	0.000	0.005
DBAcAm 0 µM vs. DBAcAm 25 µM	76.731	23.458	0.000	0.006
DBAcAm 0 µM vs. DBAcAm 20 µM	69.602	21.279	0.000	0.006
DBAcAm 0 µM vs. DBAcAm 75 µM	100.465	20.476	0.000	0.007
DBAcAm 0 µM vs. DBAcAm 15 µM	59.185	18.094	0.000	0.009
DBAcAm 0 µM vs. DBAcAm 10 µM	42.890	13.112	0.000	0.010
DBAcAm 0 µM vs. DBAcAm 7.5 µM	32.824	10.035	0.000	0.013
DBAcAm 0 µM vs. DBAcAm 6.25 µM	30.625	9.363	0.000	0.017
DBAcAm 0 µM vs. DBAcAm 5 µM	20.415	6.795	0.000	0.025
DBAcAm 0 µM vs. DBAcAm 2.5 µM	16.380	5.008	0.000	0.050

Comparison	Significant?
DBAcAm 0 µM vs. DBAcAm 50 µM	Yes
DBAcAm 0 µM vs. DBAcAm 37.5 µM	Yes
DBAcAm 0 µM vs. DBAcAm 25 µM	Yes
DBAcAm 0 µM vs. DBAcAm 20 µM	Yes
DBAcAm 0 µM vs. DBAcAm 75 µM	Yes
DBAcAm 0 µM vs. DBAcAm 15 µM	Yes
DBAcAm 0 µM vs. DBAcAm 10 µM	Yes
DBAcAm 0 µM vs. DBAcAm 7.5 µM	Yes
DBAcAm 0 µM vs. DBAcAm 6.25 µM	Yes
DBAcAm 0 µM vs. DBAcAm 5 µM	Yes
DBAcAm 0 µM vs. DBAcAm 2.5 µM	Yes

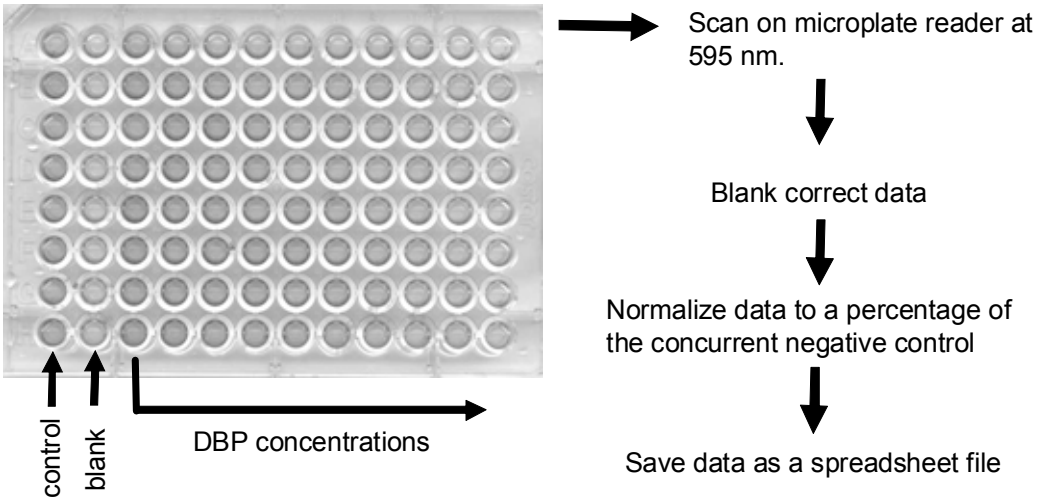


Figure 2.1 A stained microplate illustrating the CHO cell chronic cytotoxicity assay. The control (column 1) contained cells not exposed to the test compound. The blank column without cells was used to determine the absorbancy of the crystal violet histological dye that was not associated with cells and to normalize the absorbancy data. The DBP or test chemical was assayed from low concentration (column 3) to high concentration (column 12) with 8 replicate cultures per concentration.

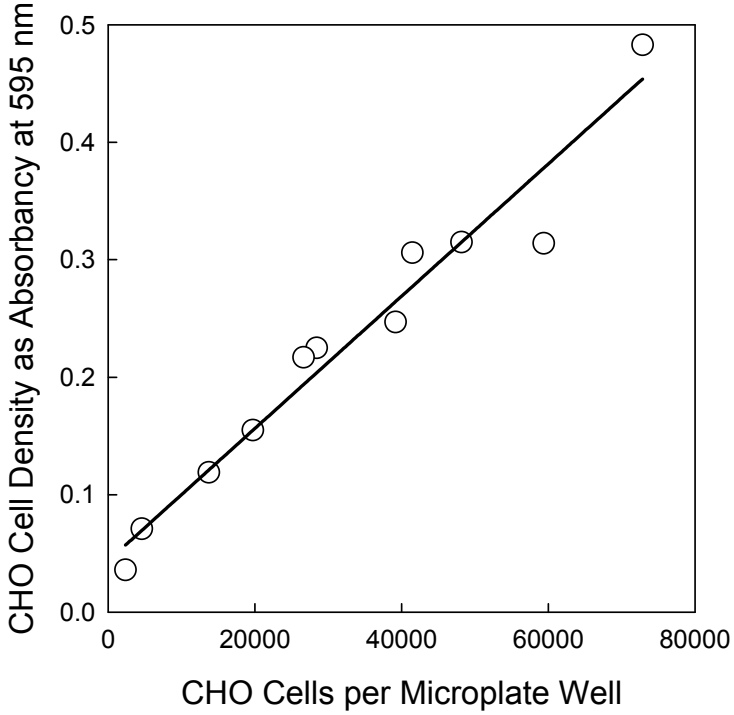


Figure 2.2 Calibration of the CHO cell cytotoxicity assay. A comparison of the number of cells per microplate well determined by Coulter counting or by the absorbancy after crystal violet staining.

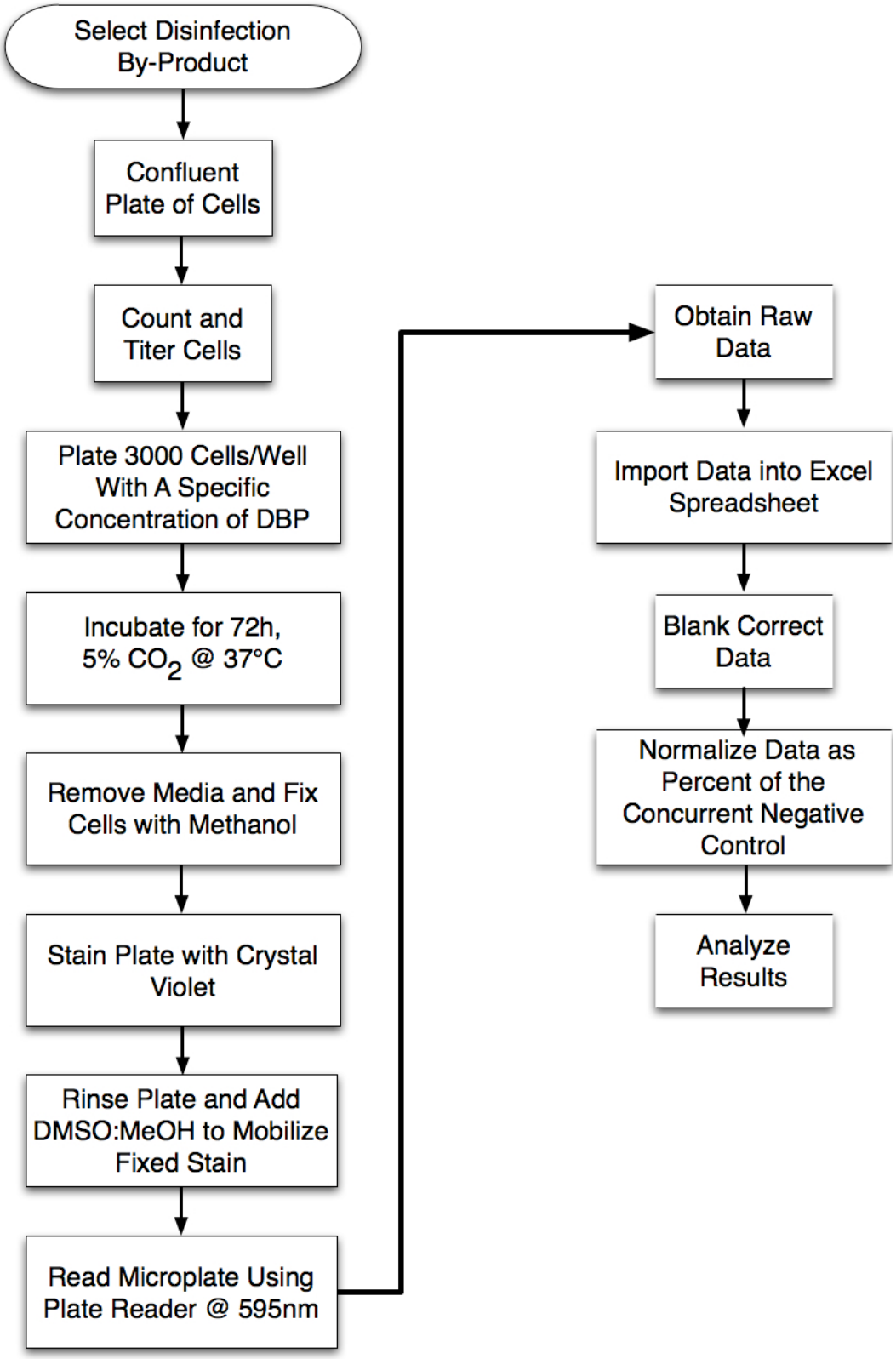


Figure 2.3 Flowchart of the CHO cell chronic cytotoxicity assay

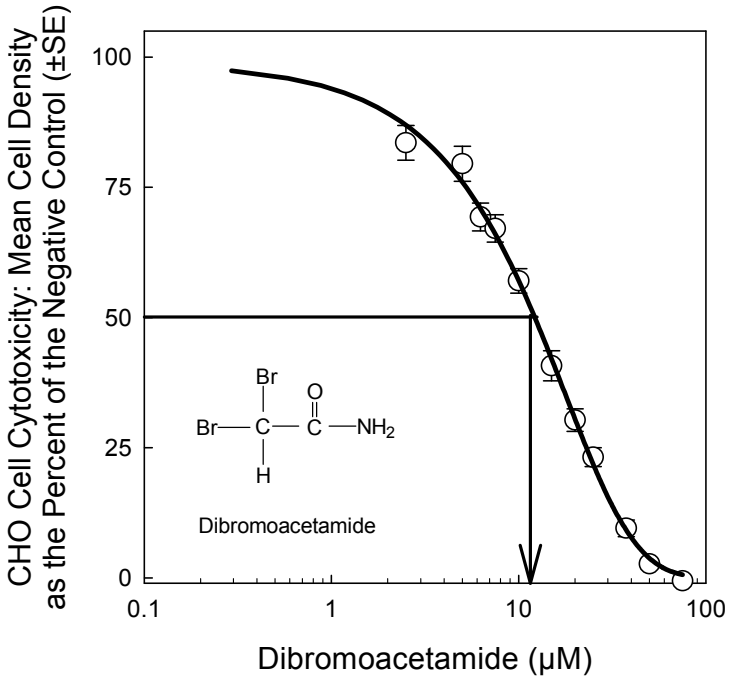


Figure 2.4 CHO cell chronic cytotoxicity concentration-response curve for dibromoacetamide illustrating the determination of the %C½ value. This value is the calculated DBP concentration (based upon regression of the concentration-response data) that induced a cell density that was 50% of the negative control and is analogous to the LC_{50} value (Plewa, Muellner et al. 2008). The R^2 from the regression analysis was 0.99 and the %C½ value = 12.2 µM.

CHO Single Cell Gel Electrophoresis (SCGE) Assay

SCGE is a molecular genetic assay that can quantitatively measure the level of genomic DNA damage induced in individual nuclei of cells (Rundell, Wagner, and Plewa 2003; Tice et al. 2000; Fairbairn, Olive, and O'Neill 1995). The day before treatment, 4×10^4 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™. With each experiment a negative control, a positive control (3.8 mM ethyl methanesulfonate, EMS) and 9 concentrations of a specific DBP were conducted concurrently. 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. To measure acute cytotoxicity a 10 µL aliquot of cell suspension was mixed with 10 µL of 0.05% trypan blue vital dye in phosphate-buffered saline (PBS) (Phillips 1973). SCGE data were not used if the acute cytotoxicity exceeded 30%. Prior to the experiment clear microscope slides were coated with a layer of 1% normal melting point agarose prepared with deionized water and dried overnight. After cell treatment, the cell suspension from each well was embedded in a layer of low melting point agarose prepared with PBS and placed upon the slides. After the microgels solidified on a tray placed over ice, a final layer of 0.5% low melting point agarose was placed upon the previous layers. 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 removed from the tank, 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 microscopic analysis the microgels were hydrated in cold water for 20-30 min and stained with 65 µL of ethidium bromide (20 µg/mL) for 3 min. The microgels were rinsed in cold water and were analyzed with a Zeiss fluorescence microscope with an excitation filter of 546/10 nm and a barrier filter of 590 nm. For each experiment 2 microgels were prepared per treatment group. Twenty-five randomly chosen nuclei were analyzed in each microgel 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 tail moment (integrated value of migrated DNA density multiplied by the migration distance) of the nuclei as a measure of DNA damage. The digitalized data (Figure 2.5) were automatically transferred to a computer based spreadsheet for subsequent statistical analysis. The experiments were repeated 3 times for each DBP. A flow diagram of the CHO SCGE assay is presented in Figure 2.6.

Normalization of CHO Genotoxicity Data and Statistical Analysis

The SCGE tail moment data for each nucleus for each microgel was generated using the Komet 3.1 software. These data were stored on a spreadsheet and the median tail moment value for that microgel was calculated and transferred to a data spreadsheet. In addition the acute cytotoxicity of the treated cells was entered into the same data spreadsheet. Table 2.7 presents the summary data spreadsheet for dibromoacetamide. Within the DBP concentration range that allowed for 70% or greater viable cells, a concentration-response curve was generated. The data were plotted and a regression analysis was used to fit the curve (Figure 2.7). The SCGE genotoxic potency value was calculated for each DBP. The SCGE genotoxic potency value was determined from the concentration-response curve. It represents the midpoint of the curve within the concentration range that expressed above 70% cell viability.

Table 2.7
Summary of CHO SCGE data for dibromoacetamide with the acute cytotoxicity measurements

Conc (µM)	Experiment 041305EW		Experiment 060905EW				Experiment 071405MM				Num	Av	SE	Live	Dead	% Via
0.0	0.247	0.204	0.192	0.307			0.027	0.181			6	0.19	0.04	126	4	96.9
25.0	0.231	0.199									2	0.21	0.02	120	13	90.2
50.0	0.178	0.614									2	0.40	0.22	117	0	100
100.0	0.594	0.707	0.318	0.102			0.178	0.056			6	0.33	0.11	132	3	97.8
250.0	13.504	5.269	3.600	3.823			8.657	5.673			6	6.75	1.54	129	3	97.7
350.0			3.850	7.249			13.060	40.805			4	16.24	8.41			
500.0	37.791	23.898	12.342	10.021			35.893	44.596			6	27.42	5.82	132	3	97.8
600.0			18.051	18.160	14.40	18.44	43.017	43.077	39.99	53.4	8	31.07	5.41			
750.0	34.941	29.257	24.679	25.340			63.135	67.183			6	40.76	7.88	128	2	98.5
1000	49.939	58.719	29.166	29.709			88.005	63.546			6	53.18	9.11	121	7	94.5
2500	74.867	77.637	73.026	78.150			87.160	87.931			6	79.80	2.57	112	8	93.3
5000	82.911	77.726									2	80.32	2.59	113	1	99.1

The data were transferred to the SigmaStat 3.1 program spreadsheet for an ANOVA statistical test (Table 2.8). The tail moment values in the SCGE assay are not normally distributed

and violate the requirements for analysis by parametric statistics. The median tail moment value for each microgel was determined as described above and the data were averaged amongst all of the microgels for each DBP concentration. Averaged median or mean values express a normal distribution according to the central limit theorem (Box, Hunter, and Hunter 1978). The averaged median tail moment values obtained from repeated experiments were analyzed with a one-way ANOVA test (Lovell, Thomas, and Dubow 1999). If a significant F value of $P \leq 0.05$ was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted. The power of the test statistic ($1-\beta$) was maintained as ≥ 0.8 at $\alpha=0.05$.

SAFETY

Safety is a principal concern in the laboratory. Manipulations of toxic, genotoxic and/or carcinogenic chemicals were conducted using disposable papers and gloves in a certified stage two containment biological/chemical safety hood. The disposal of hazardous material was in compliance with the University of Illinois regulations. All staff involved on this project received safety training under the auspices of the University of Illinois Institutional Biological Safety Committee. The regulations of the Division of Research Safety of the University of Illinois were implemented throughout this project. Experimental designs for each DBP analyzed were prepared in hard-copy data books. The information for each experiment, identified by a unique experiment number, was placed in a file within an Excel spreadsheet.

Table 2.8
Statistical analysis of the CHO cell SCGE genotoxicity of dibromoacetamide

One Way Analysis of Variance CHO CELL SCGE TAIL MOMENT
2,2-DIBROMOACETAMIDE
Data source: Data 21 in AWWARF3089 Master 071705.SNB

Group Name	N	Missing	Mean	Std Dev	SEM
DBAcAm TM 0	6	0	0.193	0.0938	0.0383
DBAcAm TM 25	2	0	0.215	0.0224	0.0159
DBAcAm TM 050	2	0	0.396	0.308	0.218
DBAcAm TM 01006		0	0.326	0.269	0.110
DBAcAm TM 02506		0	6.754	3.771	1.539
DBAcAm TM 350	4	0	16.241	16.812	8.406
DBAcAm TM 500	6	0	27.423	14.261	5.822
DBAcAm TM 600	8	0	31.071	15.306	5.411
DBAcAm TM 750	6	0	40.756	19.294	7.877
DBAcAm TM 10006		0	53.181	22.311	9.109
DBAcAm TM 25006		0	79.795	6.291	2.568

Source of Variation	DF	SS	MS	F	P
Between Groups	10	36458.867	3645.887	21.091	<0.001
Residual	47	8124.458	172.861		
Total	57	44583.325			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).
Power of performed test with alpha = 0.050: 1.000
Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05
Comparisons for factor:

(continued)

Table 2.8 (Continued)
Statistical analysis of the CHO cell SCGE genotoxicity of dibromoacetamide

One Way Analysis of Variance CHO CELL SCGE TAIL MOMENT
2,2-DIBROMOACETAMIDE
Data source: Data 21 in AWWARF3089 Master 071705.SNB

Group Name	N	Missing	Mean	Std Dev	SEM

Comparison	Diff of Means	t	Unadjusted P	Critical Level
DBAcAm TM 0 vs. DBAcAm TM 2500	79.602	10.487	0.000	0.005
DBAcAm TM 0 vs. DBAcAm TM 1000	52.987	6.980	0.000	0.006
DBAcAm TM 0 vs. DBAcAm TM 750	40.563	5.344	0.000	0.006
DBAcAm TM 0 vs. DBAcAm TM 600	30.878	4.349	0.000	0.007
DBAcAm TM 0 vs. DBAcAm TM 500	27.230	3.587	0.001	0.009
DBAcAm TM 0 vs. DBAcAm TM 350	16.048	1.891	0.065	0.010
DBAcAm TM 0 vs. DBAcAm TM 0250	6.561	0.864	0.392	0.013
DBAcAm TM 0 vs. DBAcAm TM 050	0.203	0.0189	0.985	0.017
DBAcAm TM 0 vs. DBAcAm TM 0100	0.133	0.0175	0.986	0.025
DBAcAm TM 0 vs. DBAcAm TM 25	0.0217	0.00202	0.998	0.050

Comparison	Significant?
DBAcAm TM 0 vs. DBAcAm TM 2500	Yes
DBAcAm TM 0 vs. DBAcAm TM 1000	Yes
DBAcAm TM 0 vs. DBAcAm TM 750	Yes
DBAcAm TM 0 vs. DBAcAm TM 600	Yes
DBAcAm TM 0 vs. DBAcAm TM 500	Yes
DBAcAm TM 0 vs. DBAcAm TM 350	No
DBAcAm TM 0 vs. DBAcAm TM 0250	No
DBAcAm TM 0 vs. DBAcAm TM 050	No
DBAcAm TM 0 vs. DBAcAm TM 0100	No
DBAcAm TM 0 vs. DBAcAm TM 25	No

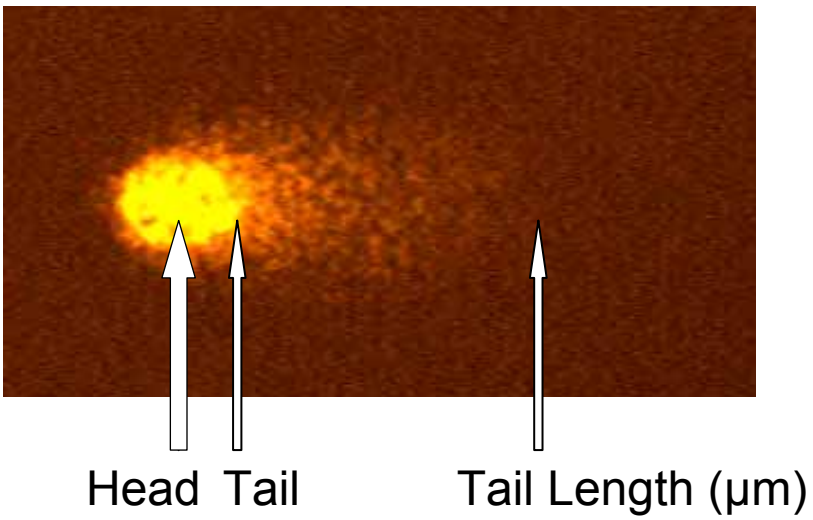


Figure 2.5 SCGE image illustrating genomic DNA damage in a nucleus. The level of DNA damage is directly related to the amount of DNA that migrates in the microgel (tail). The arrows aid the computer program for the assay that measures the amount and distance of DNA migration.

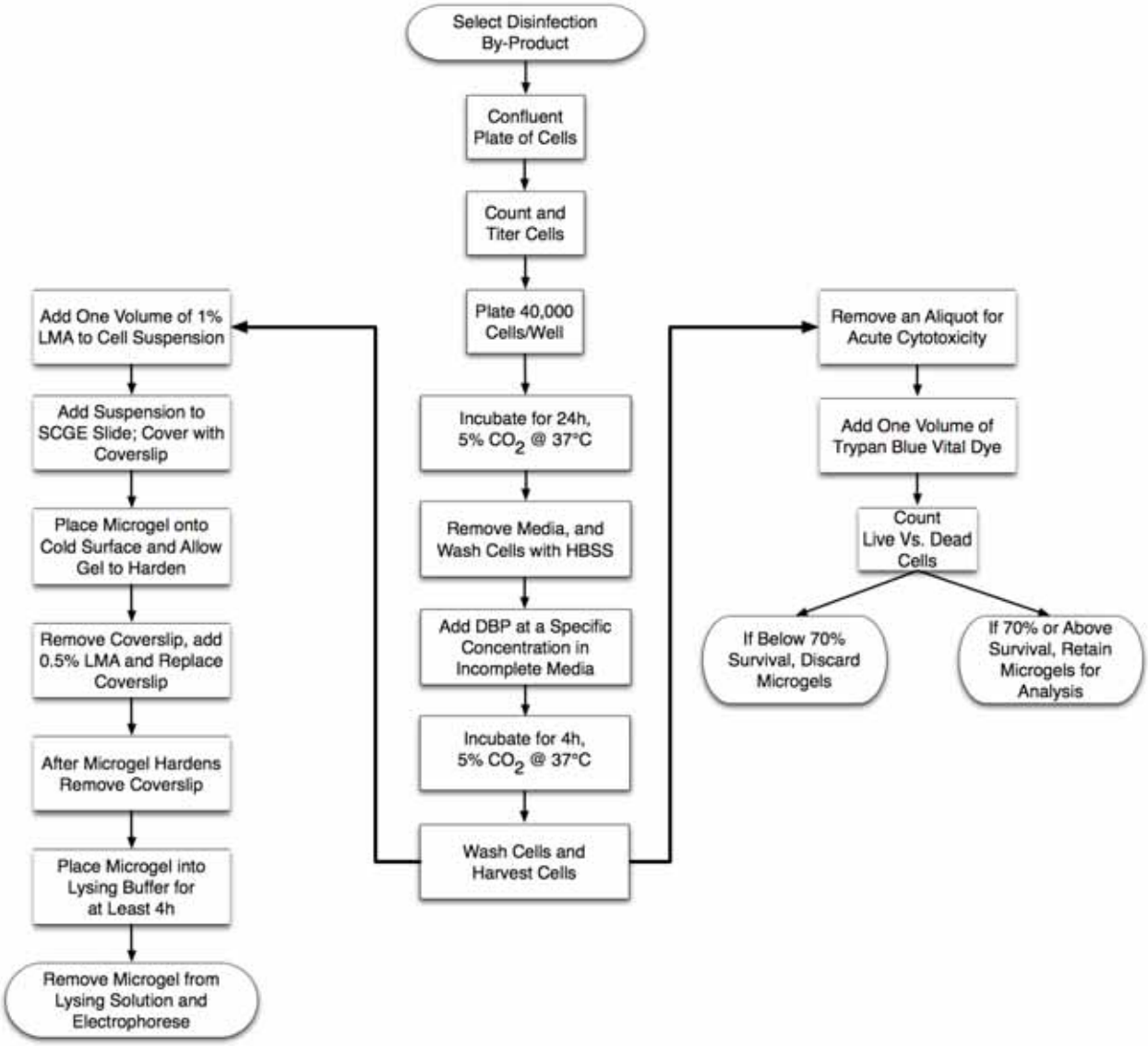


Figure 2.6. Flowchart of the CHO cell SCGE assay to measure genomic DNA damage

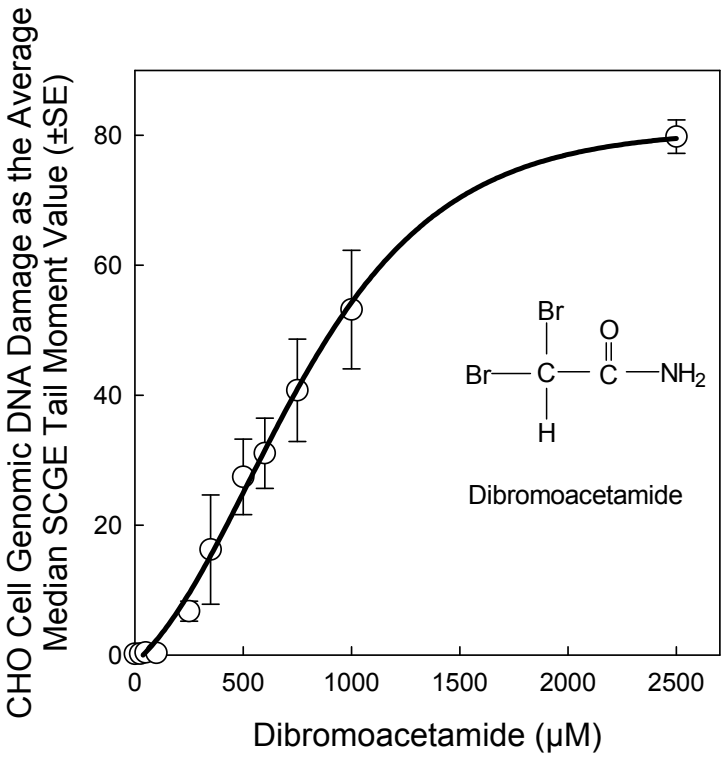


Figure 2.7 CHO cell SCGE genotoxicity concentration-response curve for dibromoacetamide

CHAPTER 3
HALOMETHANES

INTRODUCTION

In alphabetical order, the seven halomethanes analyzed in this study were bromochloroiodomethane, bromodichloromethane, chlorodibromomethane, dibromoiodomethane, tribromomethane (bromoform), trichloromethane (chloroform), and triiodomethane (iodoform). For each chemical class, the CHO cell chronic cytotoxicity data are followed by the SCGE genotoxicity data and a discussion on the structure activity relationships for the agents within each class.

OCCURRENCE

Within the halomethane class of DBPs are the trihalomethanes (THMs), chloroform, bromoform, bromodichloromethane, and chlorodibromomethane that are regulated by the U.S. EPA at 80 µg/L, total trihalomethanes (U. S. Environmental Protection Agency 2006). The U.S. EPA Information Collection Rule reported mean levels of 28.0 µg/L and 90th percentile levels of 60.2 µg/L for the sum of the four regulated trihalomethanes (McGuire, McLain, and Obolensky 2002). Chloroform had the highest mean concentration of 23 µg/L. Trihalomethanes constitute the predominant DBP class formed in chlorinated drinking water (Krasner et al. 2006). These DBPs are formed at much lower levels in chloraminated drinking water. However, bromoform can be formed in high-bromide source waters treated with ozone (Glaze and Weinberg 1993; Richardson 1998). Bromodichloromethane, chlorodibromomethane, and bromoform can increase in formation relative to chloroform when elevated levels of natural bromide are present in source waters. Disinfection with chlorine dioxide does not form trihalomethanes; however, low levels can be present due to chlorine impurities in chlorine dioxide.

GENOTOXICITY

Some of the halomethanes have been studied intensively over the past 30 years, and many *in vitro* and *in vivo* methods have been used to investigate their mutagenic and genotoxic properties (IARC 1999). In this report the term "mutagenicity" refers to assays that measure a change in DNA sequence (either gene or chromosomal mutation); the term "genotoxicity" refers to mutagenicity as well as DNA damage (DNA adducts, DNA strand breaks, etc.). Bromodichloromethane, chlorodibromomethane, and bromoform generally did not induce gene mutations in the standard test systems (IARC 1999). However, these brominated halomethanes were activated to mutagens by glutathione-*S*-transferase-theta1-1 (GSTT1-1) in *S. typhimurium* strain RSJ100; the rank order of their mutagenic potency was bromoform > bromodichloromethane > chlorodibromomethane (DeMarini et al. 1997; Pegram et al. 1997). It was demonstrated that GSTT1-1 catalyzed the covalent binding of bromodichloromethane to DNA and induced the formation of guanine adducts (Ross and Pegram 2004). With few exceptions, chloroform was not mutagenic or genotoxic in a wide array of systems and endpoints *in vivo* or *in vitro*. Although some weak positive responses were observed, these results were not repeatable (IARC 1999). Unlike other thrihalomethanes, chloroform was not activated by GSTT1-1 to a mutagen in *Salmonella* (Pegram et al. 1997).

CHO CELL CYTOTOXICITY ANALYSIS OF THE HALOMETHANES

The CHO cell chronic cytotoxicity of the 7 halomethanes analyzed in this study are presented in Table 3.1. In the table, the lowest concentration of a specific halomethane 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 halomethane 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.

Table 3.1
CHO cell chronic cytotoxicity of the halomethane DBPs and related chemicals

Compound	Lowest Tox. Conc. (M)	R^2	%C½ (M)	ANOVA Test Statistic
Bromochloroiodomethane	2.20×10^{-3}	0.95	2.42×10^{-3}	$F_{9, 155} = 12.8$; $P \le 0.001$
Bromodichloromethane	4.00×10^{-3}	0.99	1.15×10^{-2}	$F_{10, 165} = 34.7$; $P \le 0.001$
Chlorodibromomethane	7.50×10^{-4}	0.97	5.36×10^{-3}	$F_{17, 158} = 29.9$; $P \le 0.001$
Dibromoiodomethane	1.50×10^{-3}	0.92	1.90×10^{-3}	$F_{18, 102} = 42.8$; $P \le 0.001$
Tribromomethane (bromoform)	1.00×10^{-4}	0.93	3.96×10^{-3}	$F_{15, 133} = 61.95$; $P \le 0.001$
Trichloromethane (chloroform)	6.00×10^{-3}	0.92	9.62×10^{-3}	$F_{18, 325} = 12.9$; $P \le 0.001$
Triiodomethane (iodoform)	1.00×10^{-5}	0.91	6.60×10^{-5}	$F_{13, 250} = 84.2$; $P \le 0.001$

The CHO cell chronic cytotoxicity concentration-response curves are presented for each halomethane in the following figures: bromochloroiodomethane (Figure 3.1), bromodichloromethane (Figure 3.2), chlorodibromomethane (Figure 3.3), dibromoiodomethane (Figure 3.4) , tribromomethane (bromoform) (Figure 3.5), trichloromethane (chloroform) (Figure 3.6), and triiodomethane (iodoform) (Figure 3.7).

Comparative CHO Cell Chronic Cytotoxicity of the Halomethanes

A comparison of the relative cytotoxicity of the 7 halomethanes is presented in Figure 3.8. The rank order, from highest to lowest cytotoxicity, based on the %C½ values, is iodoform >> dibromoiodomethane > bromochloroiodomethane > bromoform > chlorodibromomethane > chloroform > bromodichloromethane (Table 3.1). These data indicate the cytotoxicity of these trihalomethanes follows the general order of iodo-, iodobromo-, iodobromochloro-, bromo-, followed by bromochloro- and chloro- groups. Cytotoxicity is dependent upon the halogen and its tendency as a leaving group.

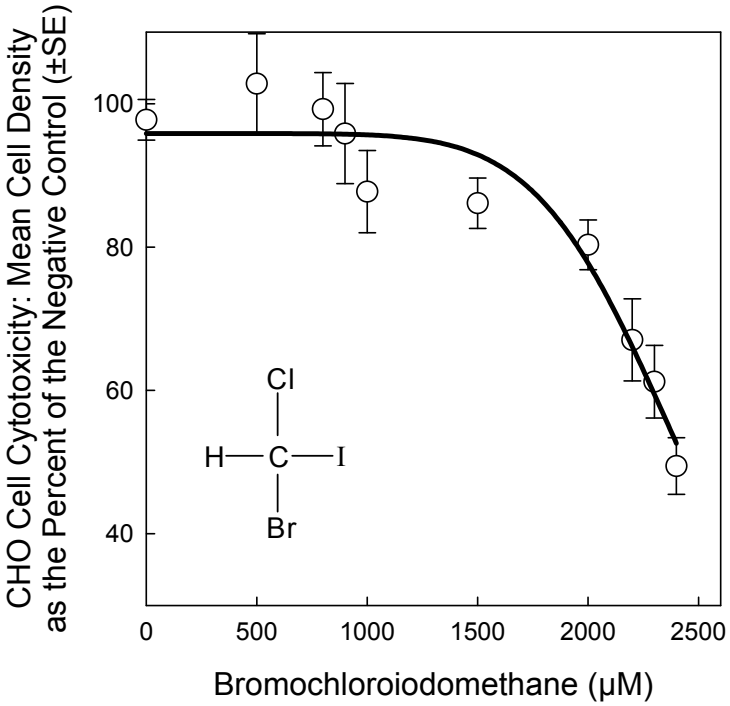


Figure 3.1 CHO cell chronic cytotoxicity concentration-response curve for bromochloroiodomethane

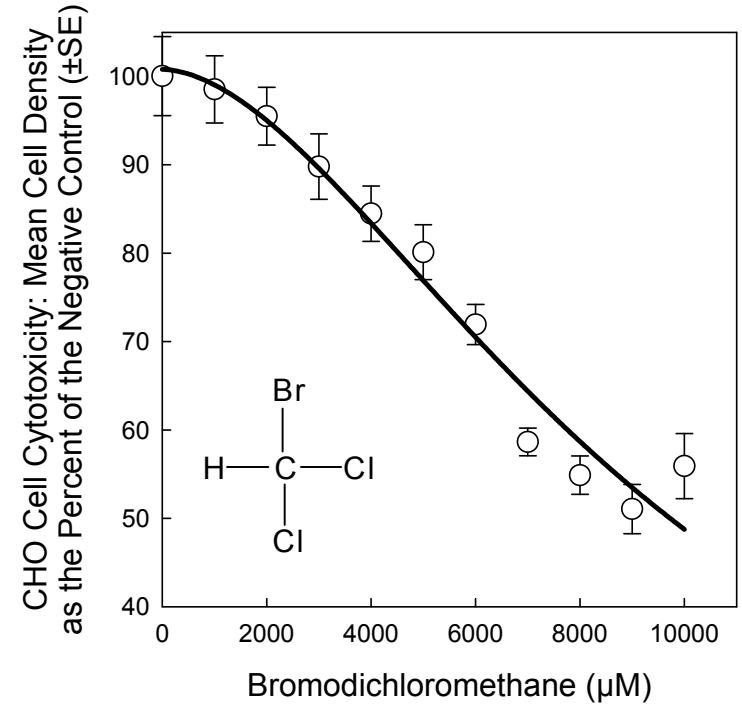


Figure 3.2 CHO cell chronic cytotoxicity concentration-response curve for bromodichloromethane

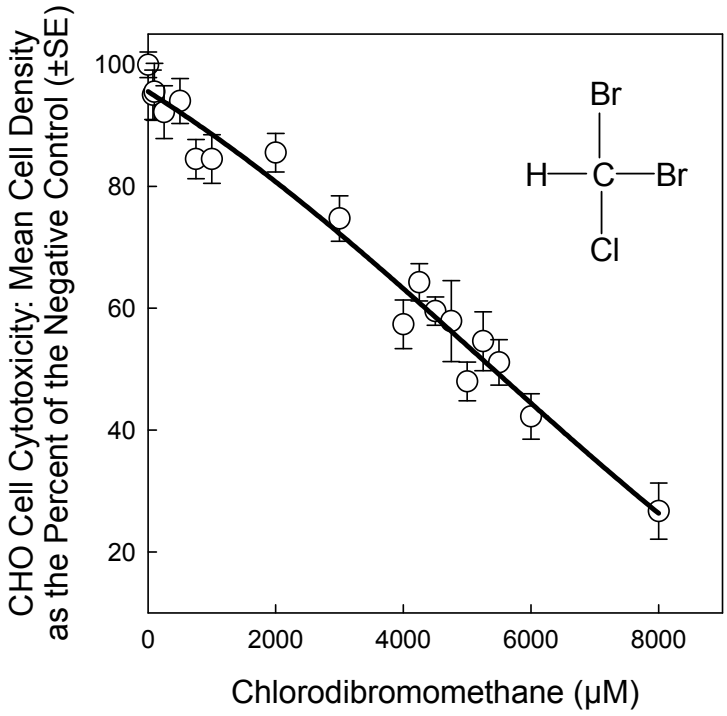


Figure 3.3 CHO cell chronic cytotoxicity concentration-response curve for chlorodibromomethane

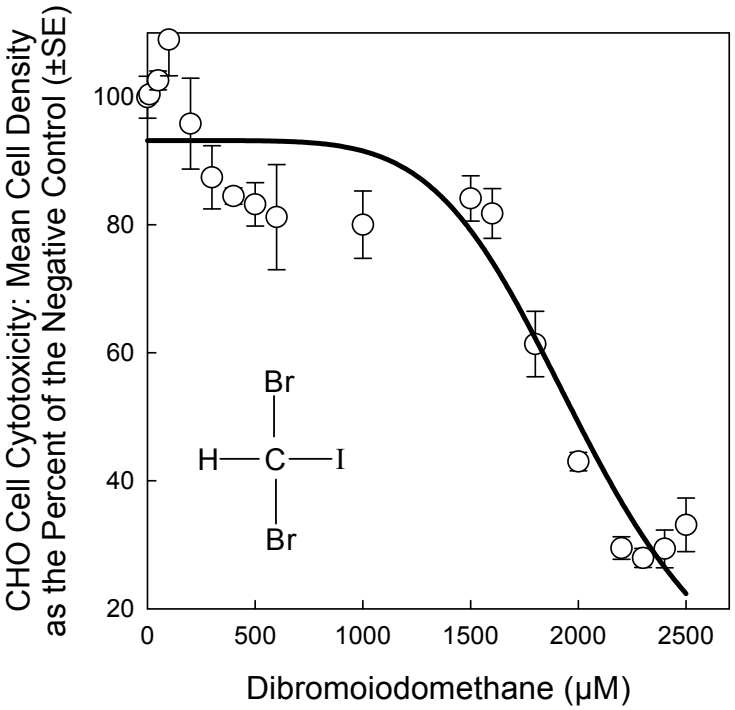


Figure 3.4 CHO cell chronic cytotoxicity concentration-response curve for dibromoiodomethane

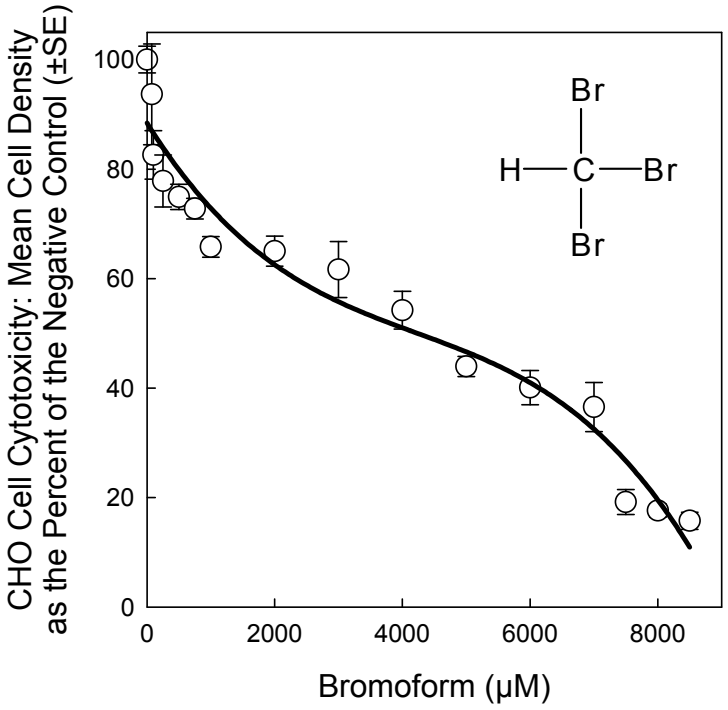


Figure 3.5 CHO cell chronic cytotoxicity concentration-response curve for tribromomethane (bromoform)

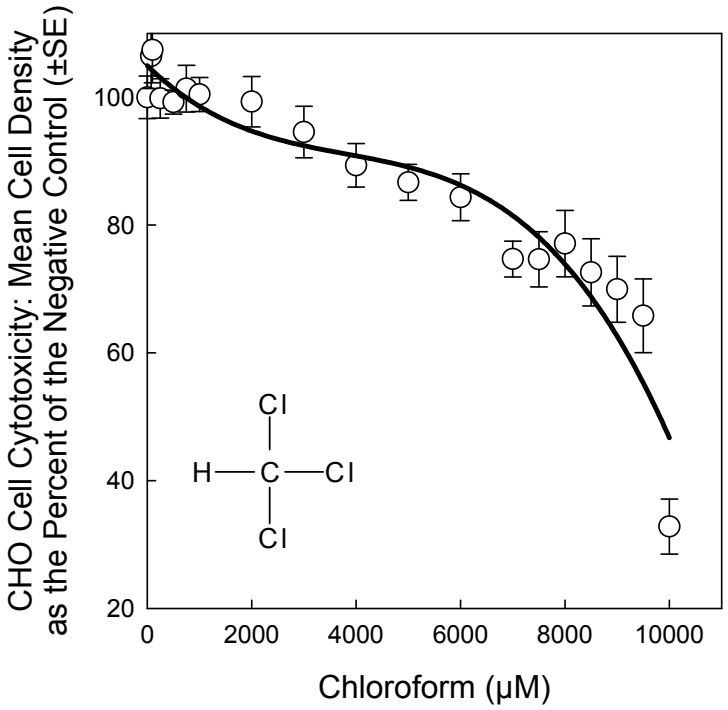


Figure 3.6 CHO cell chronic cytotoxicity concentration-response curve for trichloromethane (chloroform)

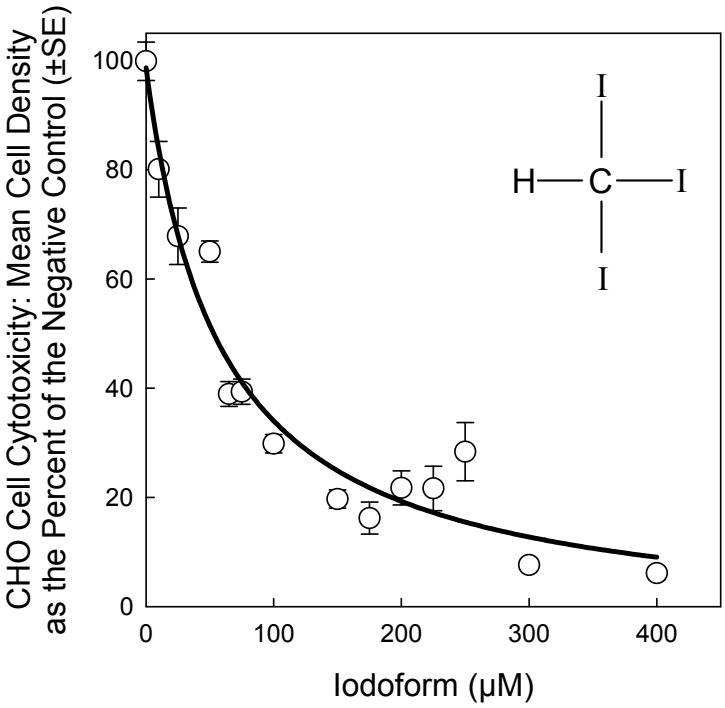


Figure 3.7 CHO cell chronic cytotoxicity concentration-response curve for triiodomethane (iodoform)

CHO CELL GENOTOXICITY ANALYSIS OF THE HALOMETHANES

Seven halomethanes were analyzed for their ability to induce genomic DNA damage in CHO cells; the results are presented in Table 3.2. In the table, the lowest concentration of a specific halomethane 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.

The data from the SCGE analysis of the halomethanes indicate that these agents were not genotoxins in this CHO cell assay (Table 3.2). No concentration-response curves are presented since there was no significant difference between treated cells and the negative controls. For all halomethanes except iodoform, the highest concentration analyzed was 10 mM. The highest concentration of iodoform analyzed was 500 μM due to acute toxicity and limited solubility. It is unknown if the CHO cell line employed in this study expressed GSTT1-1. In the *S. typhimurium* strain that expressed GSTT1-1 (RSJ100) the rank order of mutagenic potency was bromoform > bromodichloromethane > chlorodibromomethane (DeMarini et al. 1997; Pegram et al. 1997). DeMarini and his colleagues suggested that GSTT1-1-mediated metabolism which leads to mutagenic intermediates involves the removal of bromine via nucleophilic displacement or reduc-

tive dehalogenation (DeMarini et al. 1997). An interesting future study would be to incubate CHO cells with cloned GSTT1-1 with these bromomethanes to determine if GSTT1-1-mediated metabolites are genotoxic in mammalian cells.

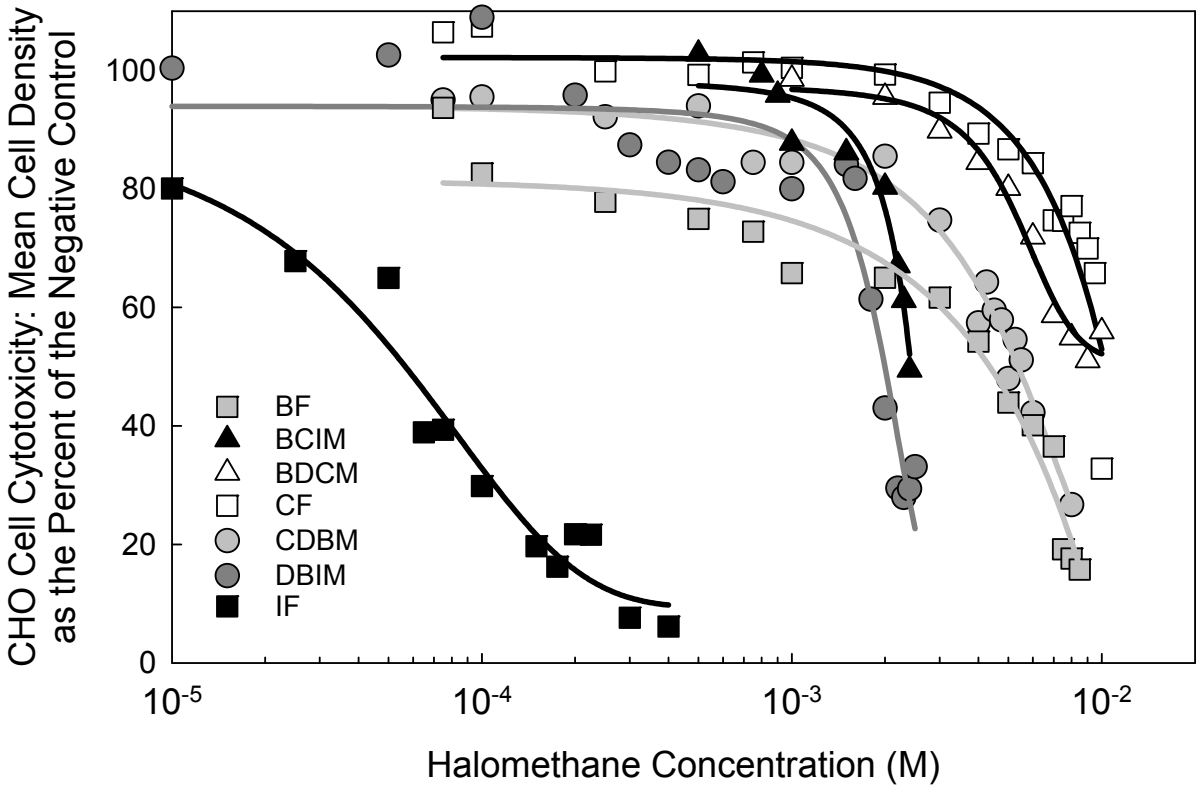


Figure 3.8 Comparison of the CHO cell chronic cytotoxicity concentration-response curves for the halomethanes analyzed in this study. The abbreviations are BF = bromoform (tribromomethane), BCIM = bromochloroiodomethane, BDCM = bromodichloromethane, CF = chloroform (trichloromethane), CDBM = chlorodibromomethane, DBIM = dibromoiodomethane, and IF = iodoform (triiodomethane)

Table 3.2
CHO cell genotoxicity of the halomethane DBPs and related chemicals

Compound	Lowest Genotox. Conc. (M)	R^2	SCGE Gen. Potency (M)	ANOVA Test Statistic
Bromochloroiodomethane	NS	–	NS	$F_{9, 30} = 0.70$; $P = 0.70$
Bromodichloromethane	NS	–	NS	$F_{9, 30} = 0.95$; $P = 0.50$
Chlorodibromomethane	NS	–	NS	$F_{9, 50} = 0.43$, $P = 0.91$
Dibromoiodomethane	NS	–	NS	$F_{9, 30} = 0.77$; $P = 0.64$
Tribromomethane (bromoform)	NS	–	NS	$F_{13, 38} = 1.07$; $P = 0.41$
Trichloromethane (chloroform)	NS	–	NS	$F_{9, 30} = 2.09$, $P = 0.06$
Triiodomethane (iodoform)	NS	–	NS	$F_{10, 39} = 0.54$; $P = 0.85$

NS = not statistically different from the negative control.

CHAPTER 4
HALOACETIC ACIDS

INTRODUCTION

The DBP class of haloacids was divided into two groups, the haloacetic acids and the haloacids with more than 2 carbon atoms (>2C-haloacids). The five haloacetic acids analyzed in this study were bromochloroacetic acid, bromodichloroacetic acid, bromoiodoacetic acid, chlorodibromoacetic acid, and diiodoacetic acid (Table 2.1).

OCCURRENCE

The five haloacetic acids regulated by the U.S. EPA are bromoacetic acid, dibromoacetic acid, chloroacetic acid, dichloroacetic acid, and trichloroacetic acid with a maximum contaminant level (MCL) of 60 µg/L for their summed concentration in drinking water (U. S. Environmental Protection Agency 2006). The highest levels of haloacetic acids are generated after chlorination but they can be formed after disinfection of water with chloramines, chlorine dioxide, and ozone (Richardson 1998). The disinfectants chloramines and chlorine dioxide form lower amounts of haloacetic acids relative to chlorine, nevertheless dichloroacetic acid, bromochloroacetic acid and dibromoacetic acids can form after chlorine dioxide treatment of raw waters (Krasner et al. 2006; Richardson 2003; Monarca et al. 2003; Zhang et al. 2000). A review of the Information Collection Rule data revealed that water utilities using chlorine dioxide as a disinfectant had higher haloacetic acid levels than those using chlorine or chloramine only (McGuire, McLain, and Obolensky 2002). In the recent Nationwide Occurrence Study the formation of the dihaloacetic acids were found (Krasner et al. 2006). Another disinfectant, ozone, that is used as an alternative to chlorine also displayed lower levels of formation of the trihalomethanes and haloacetic acids, relative to chlorine. However, with source waters containing amounts of natural bromide, dibromoacetic acid was generated with ozone disinfection (Glaze and Weinberg 1993; Richardson 1998).

The iodoacetic acids are a new group of DBPs that was identified as part of the U.S. Nationwide Occurrence Study (Krasner et al. 2006). Iodoacetic, bromoiodoacetic, and diiodoacetic acid were discovered in chloraminated drinking-water extracts. These iodoacetic acids and other iodinated haloacids and iodinated methanes may be formed at increased levels in waters treated with chloramines. These iodo-acids were detected in chloraminated drinking waters from several cities in the low ppb levels (Richardson et al. 2006).

GENOTOXICITY

A recent IARC report presented the genotoxicity data for some haloacetic acids (IARC 2004). In direct *in vitro* assays, iodoacetic acid, bromoacetic acid, dibromoacetic acid, tribromoacetic acid, and chloroacetic acid were mutagenic in *S. typhimurium* and induced genomic DNA damage (employing the SCGE assay) in CHO cells (Kargalioglu et al. 2002; Plewa et al. 2002; Plewa, Wagner, Jazwierska et al. 2004; Cemeli et al. 2006). Iodoacetic acid induced chromosomal aberrations in CHO cells (Hilliard et al. 1998). Dichloroacetic acid was weakly mutagenic in bacteria (Kargalioglu et al. 2002; DeMarini, Perry, and Shelton 1994; Giller et al. 1997). Dichloroacetic acid was not clastogenic in newts, rat bone marrow, or in mouse lymphoma cells

(Giller et al. 1997; Harrington-Brock 1998; Fox et al. 1996), but was weakly positive for inducing chromosome breaks in mice (Fuscoe et al. 1996) and at high concentrations induced mutation and chromosome aberrations in mouse lymphoma cells (Harrington-Brock 1998). Dichloroacetic acid did not induce DNA damage in CHO cells (Plewa et al. 2002) or in rodent liver cells (Chang, Daniel, and DeAngelo 1992) and was only weakly mutagenic in transgenic Big Blue mice (Leavitt et al. 1997). Trichloroacetic acid was generally negative in gene mutation tests in bacteria and mammalian cells and DNA damage assays *in vitro* (IARC 2004). However, in single studies trichloroacetic acid induced DNA damage (SCGE assay) and chromosome aberrations *in vivo* (IARC 2004). Two studies evaluated six haloacetic acids for mutagenicity in *S. typhimurium* (Giller et al. 1997; Kargalioglu et al. 2002). Although the reports listed slightly different rankings for the compounds in terms of cytotoxic and mutagenic potency, the brominated acetic acids were more toxic than their chlorinated analogues, and toxicity increased with a decrease in the number of halogen atoms per molecule. Brominated haloacetic acids also were more mutagenic than the chlorinated acids. Based on the induction of genomic DNA damage in CHO cells, the brominated haloacetic acids were more genotoxic and cytotoxic than their chlorinated analogues (Plewa et al. 2002).

CHO CELL CYTOTOXICITY ANALYSIS OF THE HALOACETIC ACIDS

The CHO cell chronic cytotoxicity of the five haloacetic acids analyzed in this study are presented in Table 4.1. In the table, the lowest concentration of a specific haloacetic acid 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 haloacetic acid 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.

Table 4.1
CHO cell chronic cytotoxicity of the haloacetic acid DBPs and related chemicals

Compound	Lowest Tox. Conc. (M)	R^2	%C½ (M)	ANOVA Test Statistic
Bromochloroacetic acid	3.00×10^{-4}	0.98	7.78×10^{-4}	$F_{10,\,165} = 49.1$; $P \le 0.001$
Bromodichloroacetic acid	5.00×10^{-4}	0.98	6.85×10^{-4}	$F_{12,\,163} = 78.7$; $P \le 0.001$
Bromoiodoacetic acid	2.50×10^{-4}	0.96	8.97×10^{-4}	$F_{20,\,239} = 59.2$; $P \le 0.001$
Chlorodibromoacetic acid	1.00×10^{-4}	0.98	2.02×10^{-4}	$F_{11,\,129} = 127.3$; $P \le 0.001$
Diiodoacetic acid	1.00×10^{-4}	0.97	3.32×10^{-4}	$F_{20,\,239} = 49.9$; $P \le 0.001$

The CHO cell chronic cytotoxicity concentration-response curves are presented for each haloacetic acid in the following figures: bromochloroacetic acid (Figure 4.1), bromodichloroacetic acid (Figure 4.2), bromoiodoacetic acid (Figure 4.3), chlorodibromoacetic acid (Figure 4.4), and diiodoacetic acid (Figure 4.5). By combining previously published data on the haloacetic acids (Plewa, Wagner et al. 2008; Plewa, Wagner, Richardson et al. 2004; Plewa et al. 2002), a more complete comparison of the CHO cell chronic cytotoxicity for 12 haloacetic acids is presented in Figure 4.6.

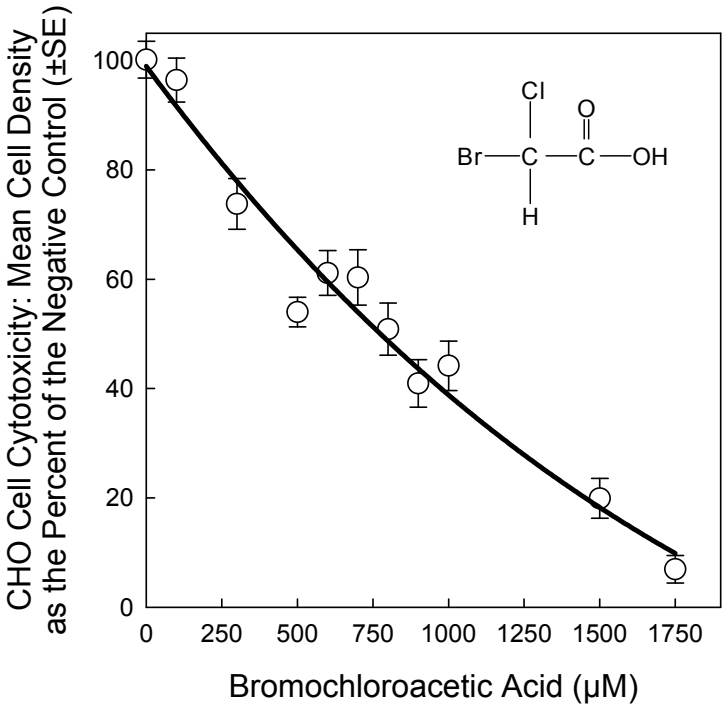


Figure 4.1 CHO cell chronic cytotoxicity concentration-response curve for bromochloroacetic acid

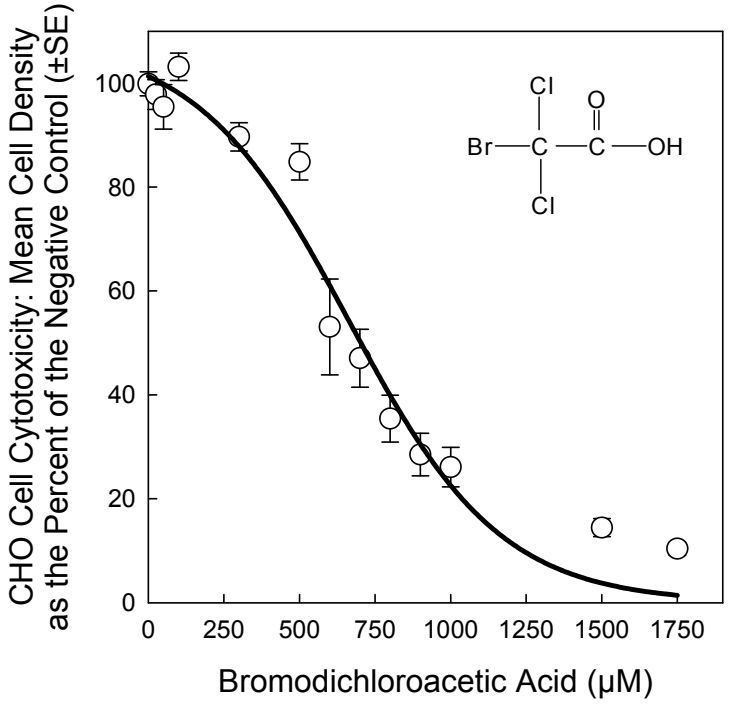


Figure 4.2 CHO cell chronic cytotoxicity concentration-response curve for bromodichloroacetic acid

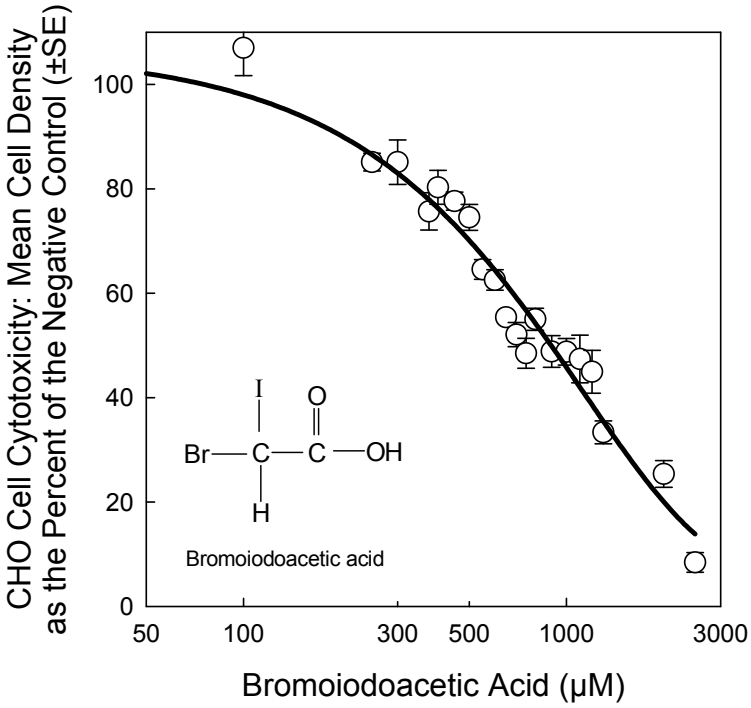


Figure 4.3 CHO cell chronic cytotoxicity concentration-response curve for bromoiodoacetic acid

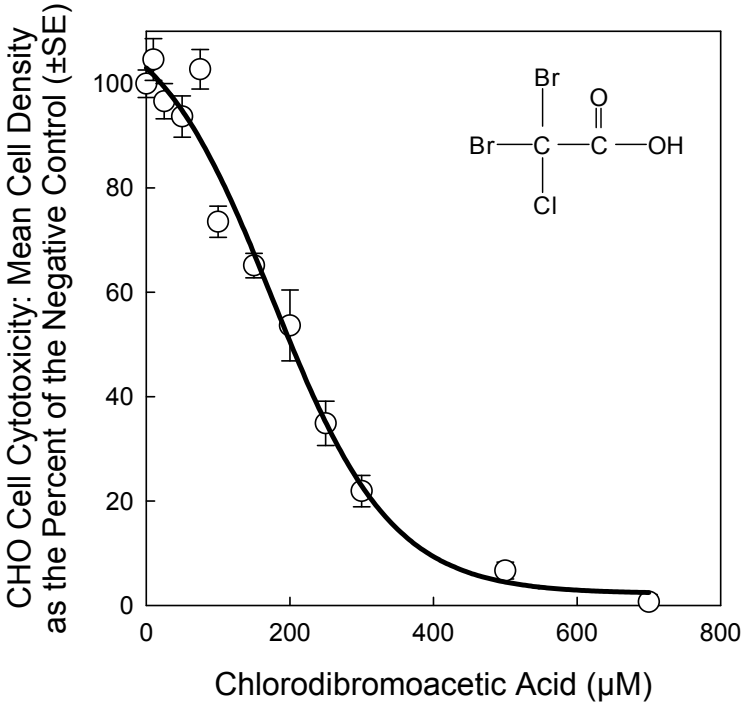


Figure 4.4 CHO cell chronic cytotoxicity concentration-response curve for chlorodibromoacetic acid

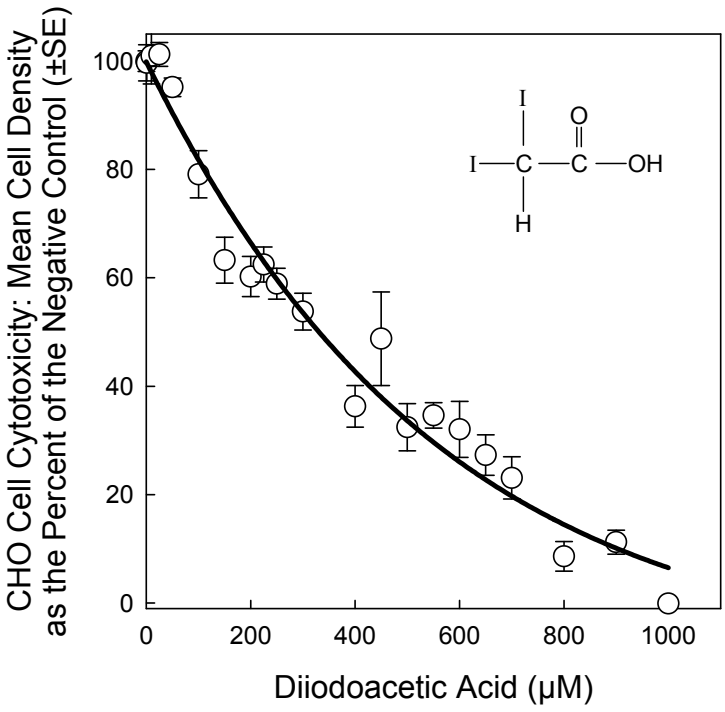


Figure 4.5 CHO cell chronic cytotoxicity concentration-response curve for diiodoacetic acid

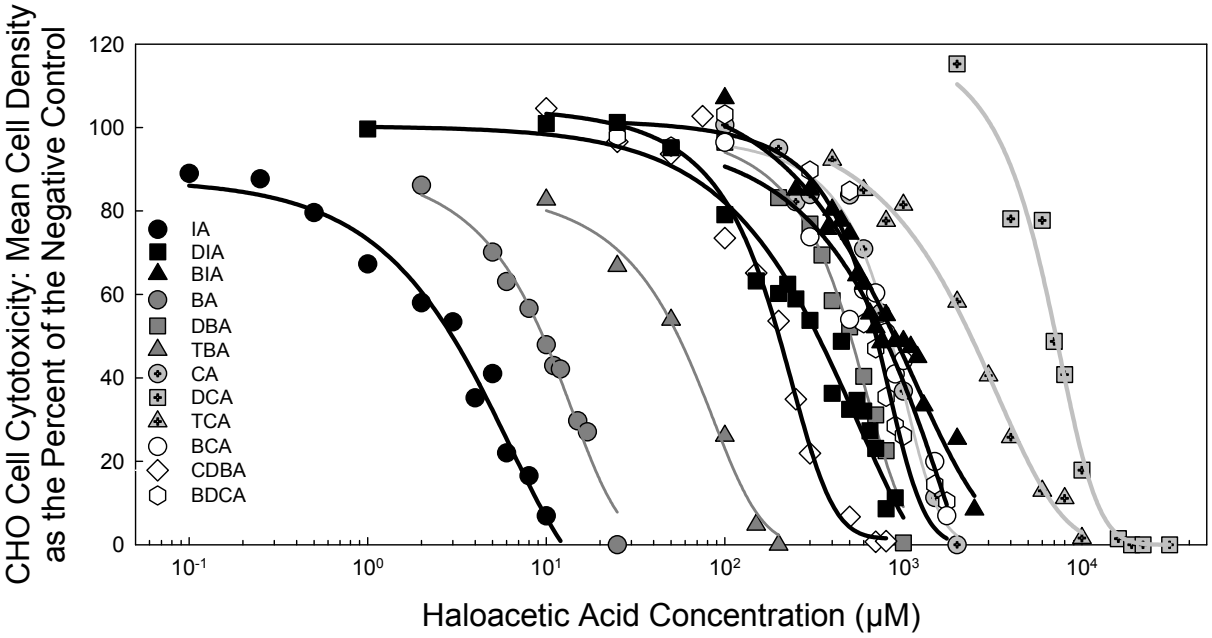


Figure 4.6 Comparison of the concentration-response curves for the CHO cell chronic cytotoxicity of 12 haloacetic acids. The abbreviations are IA = iodoacetic acid, DIA = diiodoacetic acid, BIA = bromoiodoacetic acid, BA = bromoacetic acid, DBA = dibromoacetic acid, TBA = tribromoacetic acid, CA = chloroacetic acid, DCA = dichloroacetic acid, TCA = trichloroacetic acid, BCA = bromochloroacetic acid, CDBA = chlorodibromoacetic acid, and BDCA = bromodichloroacetic acid

CHO CELL GENOTOXICITY ANALYSIS OF THE HALOACETIC ACIDS

In this study five haloacetic acids were analyzed for their ability to induce genomic DNA damage in CHO cells; the results are presented in Table 4.2. In the table, the lowest concentration of a specific haloacetic acid 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. The CHO cell concentration-response curves illustrating the induction of genomic DNA damage are presented for bromochloroacetic acid, bromodichloroacetic acid, bromoiodoacetic acid, chlorodibromoacetic acid, and diiodoacetic acid in Figures 4.7 – 4.11, respectively. By combining previously published data (Plewa, Wagner et al. 2008; Plewa, Wagner, Richardson et al. 2004; Plewa et al. 2002), a more complete comparison of the CHO cell genotoxicity for 12 haloacetic acids is presented in Figure 4.12.

Table 4.2
CHO cell genotoxicity of the haloacetic acid DBPs and related chemicals

Compound	Lowest Genotox. Conc. (M)	R^2	SCGE Gen. Potency (M)	ANOVA Test Statistic
Bromochloroacetic acid	3.00×10^{-3}	0.99	3.64×10^{-3}	$F_{10, 41} = 37.5$; $P \leq 0.001$
Bromodichloroacetic acid	NS	–	NS	$F_{16, 61} = 1.6$; $P = 0.08$
Bromoiodoacetic acid	2.50×10^{-3}	0.97	3.16×10^{-3}	$F_{14, 43} = 14.05$; $P \leq 0.001$
Chlorodibromoacetic acid	1.30×10^{-2}	0.98	1.36×10^{-2}	$F_{21, 68} = 2.9$; $P \leq 0.001$
Diiodoacetic acid	1.00×10^{-3}	0.97	1.98×10^{-3}	$F_{13, 60} = 13.2$; $P \leq 0.001$

NS = not statistically different from the negative control.

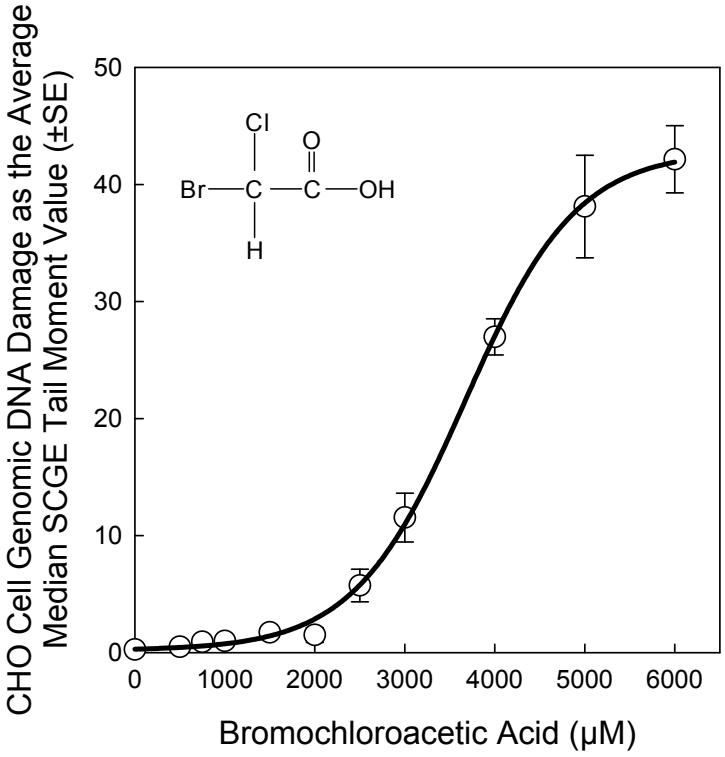


Figure 4.7 CHO cell SCGE genotoxicity concentration-response curve for bromochloroacetic acid

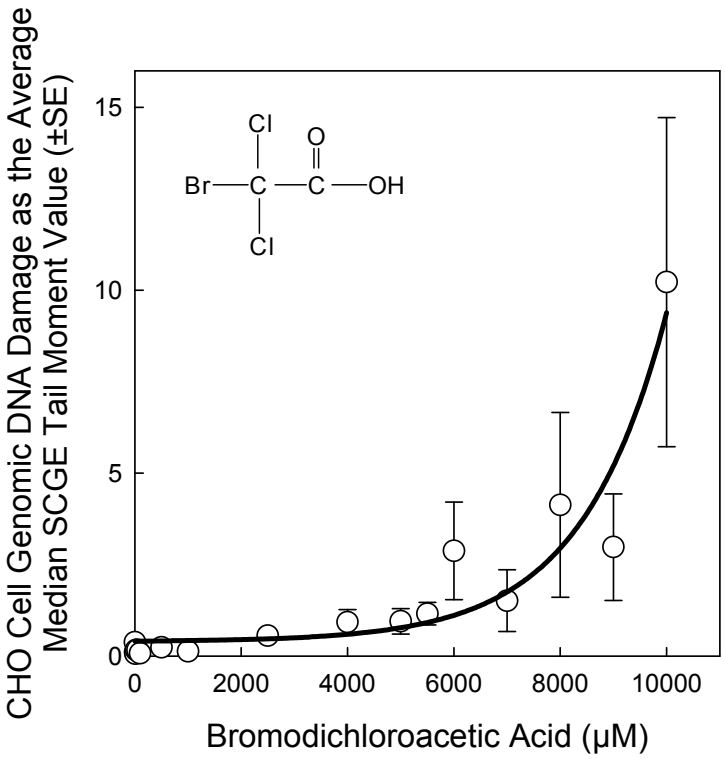


Figure 4.8 CHO cell SCGE genotoxicity concentration-response curve for bromodichloroacetic acid

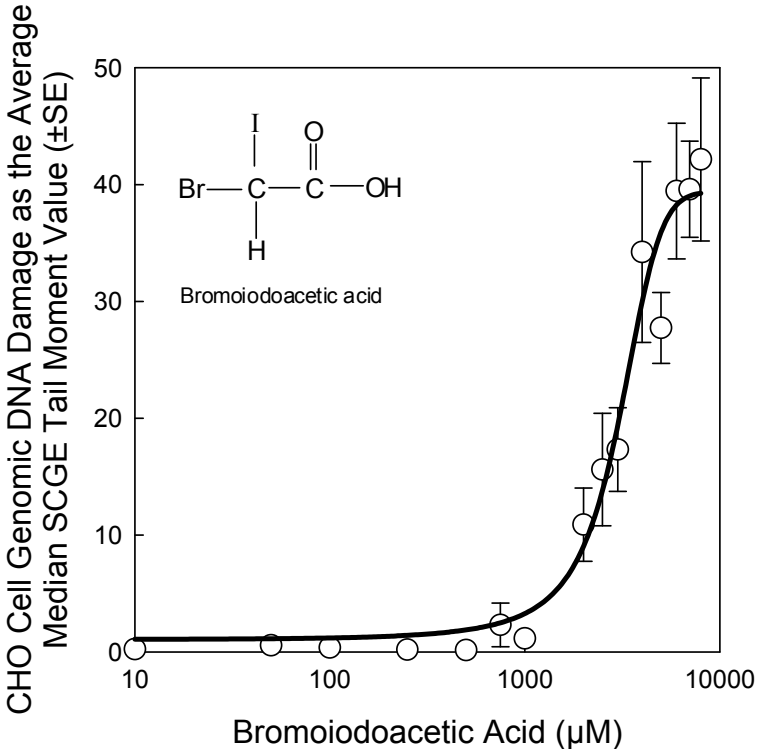


Figure 4.9 CHO cell SCGE genotoxicity concentration-response curve for bromoiodoacetic acid

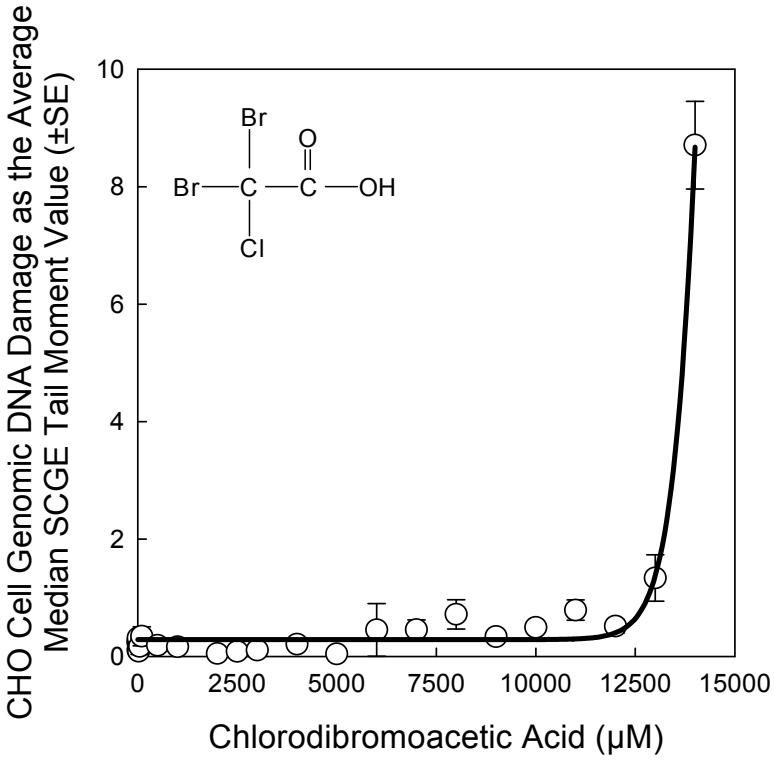


Figure 4.10 CHO cell SCGE genotoxicity concentration-response curve for chlorodibromoacetic acid

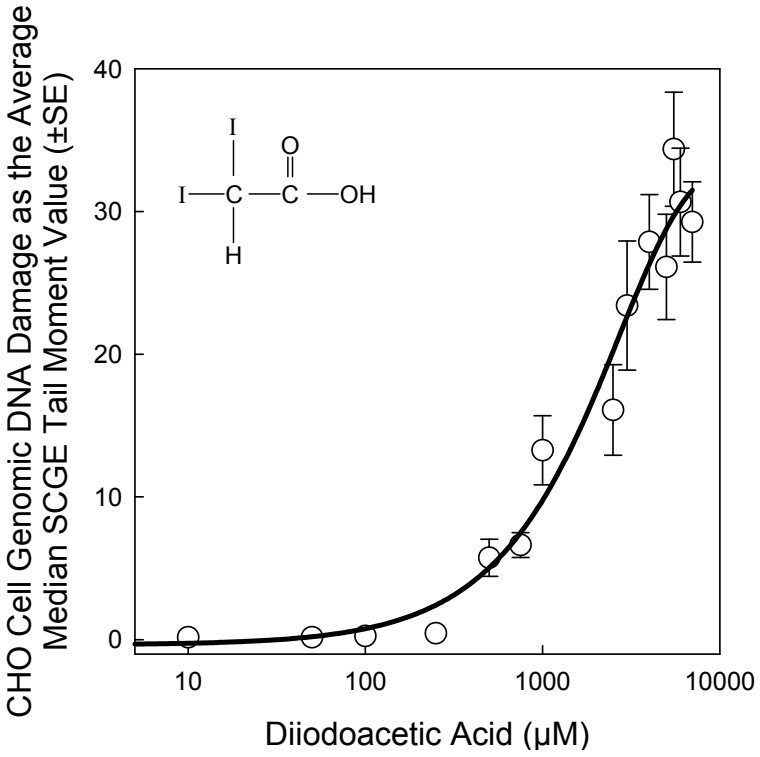


Figure 4.11 CHO cell SCGE genotoxicity concentration-response curve for diiodoacetic acid

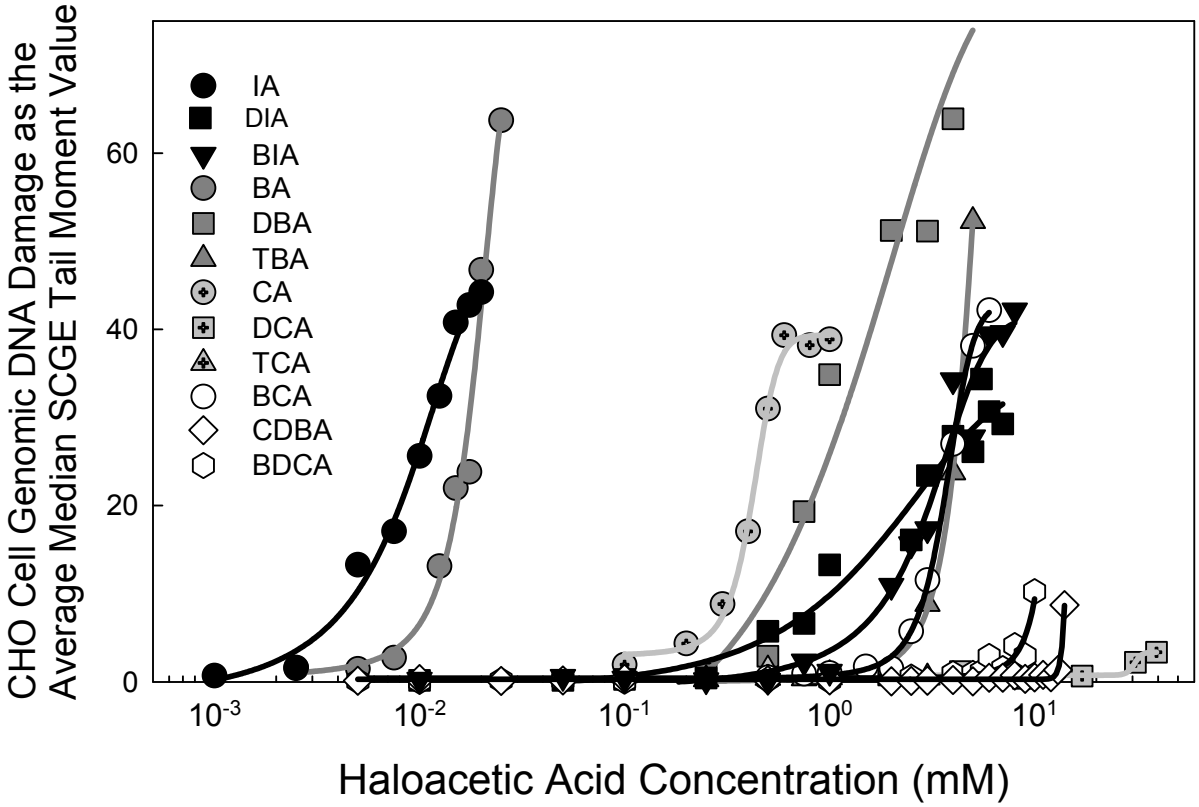


Figure 4.12 Comparison of the SCGE genotoxicity concentration-response curves of 12 haloacetic acids. The abbreviations are IA = iodoacetic acid, DIA = diiodoacetic acid, BIA = bromoiodoacetic acid, BA = bromoacetic acid, DBA = dibromoacetic acid, TBA = tribromoacetic acid, CA = chloroacetic acid, DCA = dichloroacetic acid, TCA = trichloroacetic acid, BCA = bromochloroacetic acid, CDBA = chlorodibromoacetic acid, and BDCA = bromodichloroacetic acid

COMPARATIVE CYTOTOXICITY AND GENOTOXICITY OF THE HALOACETIC ACIDS

The data from this study plus other published data constitute a set of 12 iodo-, bromo-, and chloro- haloacetic acid analogues (Figures 4.6 and 4.12) and represent the most complete systematic *in vitro* analysis of these DBPs and related compounds to date.

The data from this study and others indicate that the number and type of halogen atoms associated with the haloacetic acids have a direct impact on their *in vitro* mammalian cell toxicity (Cemeli et al. 2006; Plewa, Wagner, Richardson et al. 2004; Plewa et al. 2002, 2000). In general the mono-halogenated haloacetic acids were more cytotoxic than their di- or tri- halogenated analogues (Figure 4.6). Chloroacetic acid was approximately 12× and 19× more cytotoxic than dichloroacetic acid and trichloroacetic acid, respectively (Plewa et al. 2002). A similar pattern was observed for genomic DNA damage; the mono-halogenated agents were more genotoxic (Figure 4.12). Two important factors that govern toxicity are (i) the transport of the chemical

agent into the cell and, (ii) the reactivity of the agent with intercellular macromolecules and organelles. The data presented here suggest that as the level of ionization increases (lower p*K*a values) there is a reduced ability for the haloacetic acids to cross the cell membrane. These differences in ionization may play a role in the reduced toxicity as a function of the number of halogen atoms per haloacetic acid molecule (Plewa, Wagner, Richardson et al. 2004; Plewa et al. 2002).

In vitro cell cytotoxicity and genotoxicity of the monohaloacetic acids were studied as model compounds. Their ability to transect cell membranes is dependent on their lipophilicity, their degree of ionization, and possible transport mechanisms. Consistent with the rank order of their cytotoxicity and genotoxicity in *S. typhimurium* (Kargalioglu et al. 2002) and CHO cells (Plewa, Wagner, Richardson et al. 2004; Plewa et al. 2002), the log *P* (octanol-water partition coefficient) of the un-ionized monohaloacetic acids followed the order of iodoacetic acid > bromoacetic acid > chloroacetic acid. Increased cytotoxicity and genotoxicity was associated with log *P* values. The lipophilicity of and cell permeability to monohaloacetic acids was decreased by ionization, which is determined by their p*K*a and the pH of the medium. For the monohaloacetic acids the ranking of p*K*a followed the order of iodoacetic acid > bromoacetic acid > chloroacetic acid and was directly related to their cytotoxicity and genotoxicity. Also it is possible that facilitated or active membrane transport of anionic monohaloacetates may occur. There is evidence of the active transport of chloroacetic acid and bromoacetic acid across synthetic membranes (Yoshikawa et al. 1986) although percutaneous absorption by human skin sections demonstrated poor permeability to these haloacetic acids (Xu et al. 2002). However, under *in vivo* conditions, chloroacetic acid and iodoacetic acids accumulated in the kidneys and liver of rats (Hayes, Short, and Gibson 1973).

The chemical reactivity of monohaloacetic acids is similar to that of methyl halides which are SN2-type alkylating agents. The reactivity of methyl halides is primarily dependent on the carbon-halogen bond dissociation energy, which is related to the bond length. Since the atomic size of the halogen follows the order I > Br > Cl, the length of carbon-halogen bond increases and the bond dissociation energy decreases accordingly. Polarizability and delocalization of the electron cloud also make iodine a better leaving group than bromine and a much better leaving group than chlorine. Typically, the SN2 reactivity of an alkyl iodide is 3-5× greater than an alkyl bromide and about 50× greater than an alkyl chloride (Loudon 1995). This relative SN2 reactivity is correlated with cytotoxicity and genotoxicity in *S. typhimurium* and CHO cells (Plewa, Wagner, Richardson et al. 2004). The CHO cell cytotoxicity and genotoxicity of the monohaloacetic acids also showed a direct relationship with the calculated lowest unoccupied molecular orbital (*E*LUMO) and this supports the view that electrophilic reactivity played an important role (Plewa, Wagner, Richardson et al. 2004).

The monohaloacetic acids are soft electrophiles, which preferentially react with soft nucleophiles, such as thiol groups of cysteinyl residues in proteins and glutathione (Woo, Arcos, and Lai 1988). Some of the biological effects of the haloacetic acids may be partially due to depletion of cellular glutathione (Hayes, Short, and Gibson 1973; Chamberlain et al. 1999) which is a protective nucleophile against cytotoxicity, oxidative stress, and electrophilic attacks and a key regulator for the induction of stress-activated signal transduction pathways (Wilhelm et al. 1997).

Of special interest is a series of studies where haloacetic acids were evaluated for their ability to induce neural tube defects in a mouse embryo assay under *ex vivo* conditions (Hunter and Tugman 1995; Richard and Hunter 1996; Hunter et al. 1996). The benchmark concentration was the lower limit of the 95% confidence interval (μM concentration) of haloacetic acid that produced a 5% increase in the number of mouse embryos with neural tube defects. The haloacet-

ic acids compared were, iodoacetic acid, bromo-, dibromo-, tribromoacetic acid, chloro-, dichloro-, and trichloroacetic acid. A strong, significant correlation was found between the data for the induction of mouse neural tube defects and CHO cell chronic cytotoxicity ($r = 0.82$; $P < 0.02$). Similarly for five haloacetic acids, (iodoacetic, bromo-, dibromo-, tribromo-, and chloroacetic acids) a good correlation coefficient was observed ($r = 0.83$) between the CHO cell SCGE genotoxic potency value versus the benchmark concentrations for the induction of mouse neural tube damage, although the sample size limited our resolution and the analysis was not significant ($P = 0.08$). The conclusion from these comparisons within a single DBP chemical class is that the *in vitro* CHO cell chronic cytotoxicity and SCGE genotoxicity assays correlate quite well with an *ex vivo* mouse embryo assay. These data indicate that a prediction of toxic potency from cellular *in vitro* assays to an *ex vivo* intact animal assay is robust and positive.

CHAPTER 5
HALOACIDS

INTRODUCTION

The haloacids with more than two carbon atoms (>2C-haloacids) evaluated in this study included five with 3 carbon atoms: 3-bromo-3-iodopropenoic acid, 2,3-dibromopropenoic acid, 3,3-dibromopropenoic acid, 2-iodo-3-bromopropenoic acid, and 2,3,3-tribromopropenoic acid, one haloacid with 4 carbon atoms: 2-bromobutenedioic acid, and four haloacids with 5 carbon atoms: 3-bromo-3-chloro-4-oxopentanoic acid, 3,3-dibromo-4-oxopentanoic acid, (*E*)-2-iodo-3-methylbutenedioic acid, and *trans*-2-bromo-3-methylbutenedioic acid (Table 2.1).

OCCURRENCE

A number of emerging >2C-haloacids have been identified in finished drinking water including (*Z*)-3-bromo-3-iodopropenoic acid, (*E*)-3-bromo-3-iodopropenoic acid, and (*E*)-2-iodo-3-methylbutenedioic acid (Krasner et al. 2006). These and other >2C-haloacids can be generated in drinking water, mainly with chlorine and chloramine disinfection. One agent, 3,3-dichloropropenoic acid, was measured in the U.S. Nationwide Occurrence Study at a maximum of 4.7 µg/L. The corresponding brominated analogue, 3,3-dibromopropenoic acid, as well as several other 3-, 4-, and 5-carbon acids and di-acids were found in finished drinking water (Krasner et al. 2006). Two unusual bromo-oxo-acids, 3,3-dibromo-4-oxopentanoic acid and 3-bromo-3-chloro-4-oxopentanoic acid were also identified (Richardson et al. 2006).

Chloramination has become a widely used alternative to chlorination for water-treatment systems to comply with the Stage 2 D/DBP Rule (U. S. Environmental Protection Agency 2006). The formation of iodinated DBPs is favored in chloraminated drinking water versus chlorination due to the slower rate of oxidation to iodate and the accumuluation of hypoiodous acid (HOI) (Figure 5.1) (Bichsel and von Gunten 2000, 1999).

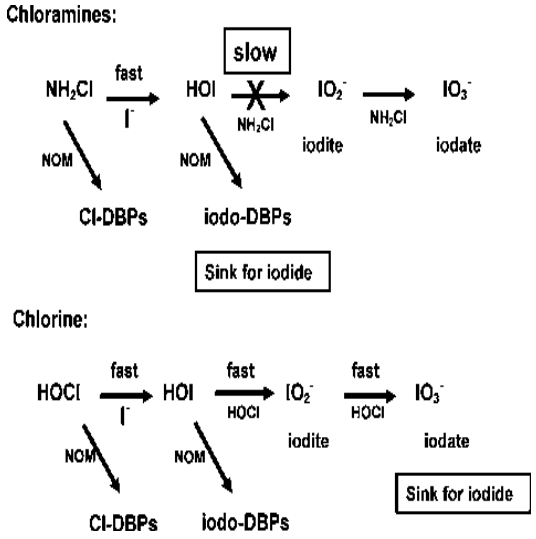


Figure 5.1 Formation of iodinated DBPs after disinfection of raw water by chloramines or chlorine (Bichsel and von Gunten 2000).

GENOTOXICITY

There is virtually no genotoxicity data available on the >2C-haloacids except that which has been generated by this study.

CHO CELL CYTOTOXICITY ANALYSIS OF THE >2C-HALOACIDS

The CHO cell chronic cytotoxicity of the ten >2C-haloacids analyzed in this study are presented in Table 5.1. In the table, the lowest concentration of a specific haloacid 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 haloacid that induced a 50% reduction of the cell density as compared to the negative controls. 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.

Table 5.1
CHO cell chronic cytotoxicity of the >2C-haloacid DBPs and related chemicals

Compound	Lowest Tox. Conc. (M)	R^2	%C½ (M)	ANOVA Test Statistic
3-Bromo-3-iodopropenoic acid	7.50×10^{-5}	0.95	1.89×10^{-4}	$F_{13,\ 184} = 78.01$; $P \leq 0.001$
2,3-Dibromopropenoic acid	1.00×10^{-3}	0.98	2.20×10^{-3}	$F_{16,\ 167} = 82.15$; $P \leq 0.001$
3,3-Dibromopropenoic acid	2.50×10^{-5}	0.97	2.95×10^{-4}	$F_{11,\ 178} = 71.82$; $P \leq 0.001$
2-Iodo-3-bromopropenoic acid	1.75×10^{-5}	0.98	4.36×10^{-5}	$F_{13,\ 137} = 106.84$; $P \leq 0.001$
2,3,3-Tribromopropenoic acid	7.50×10^{-4}	0.97	1.64×10^{-3}	$F_{15,\ 170} = 126.64$; $P \leq 0.001$
3-Bromo-3-chloro-4-oxopentanoic acid	1.0×10^{-5}	0.96	2.89×10^{-5}	$F_{11,180} = 149.1$; $P \leq 0.001$
3,3-Dibromo-4-oxopentanoic acid	5.00×10^{-6}	0.98	1.64×10^{-5}	$F_{10,\ 147} = 93.84$; $P \leq 0.001$
2-Bromobutenedioic acid	1.00×10^{-3}	0.96	2.06×10^{-3}	$F_{17,\ 166} = 62.46$; $P \leq 0.001$
(*E*)-2-Iodo-3-methylbutenedioic acid	7.00×10^{-4}	0.98	9.44×10^{-4}	$F_{11,\ 255} = 55.2$; $P \leq 0.001$
2-Bromo-3-methylbutenedioic acid	4.80×10^{-3}	0.94	5.27×10^{-3}	$F_{10,\ 84} = 12.75$; $P \leq 0.001$

The CHO cell chronic cytotoxicity concentration-response curves are presented for each >2C-haloacid in the following figures: 3-bromo-3-iodopropenoic acid (Figure 5.2), 2,3-dibromopropenoic acid (Figure 5.3), 3,3-dibromopropenoic acid (Figure 5.4), 2-iodo-3-bromopropenoic acid (Figure 5.5), 2,3,3-tribromopropenoic acid (Figure 5.6), 3-bromo-3-chloro-4-oxopentanoic acid (Figure 5.7), 3,3-dibromo-4-oxopentanoic acid (Figure 5.8), 2-bromobutenedioic acid (Figure 5.9), (*E*)-2-iodo-3-methylbutenedioic acid (Figure 5.10), and 2-bromo-3-methylbutenedioic acid (Figure 5.11). A comparison of the relative cytotoxicity of the >2C-haloacids is presented in Figure 5.12.

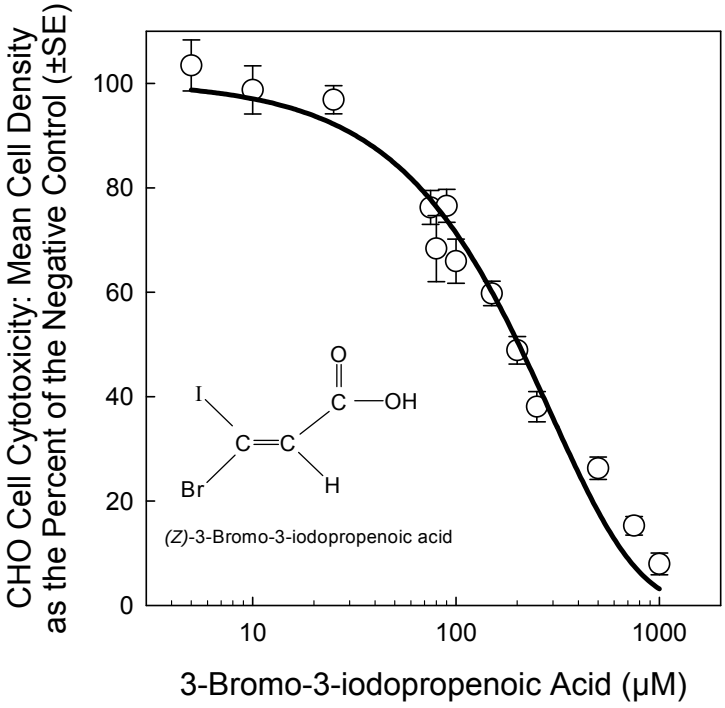


Figure 5.2 CHO cell chronic cytotoxicity concentration-response curve for 3-bromo-3-iodopropenoic acid

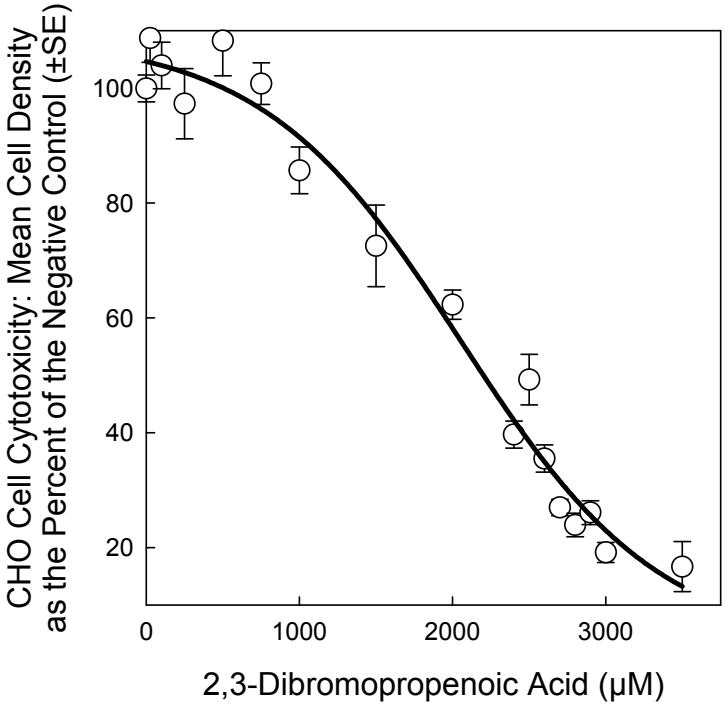


Figure 5.3 CHO cell chronic cytotoxicity concentration-response curve for 2,3-dibromopropenoic acid

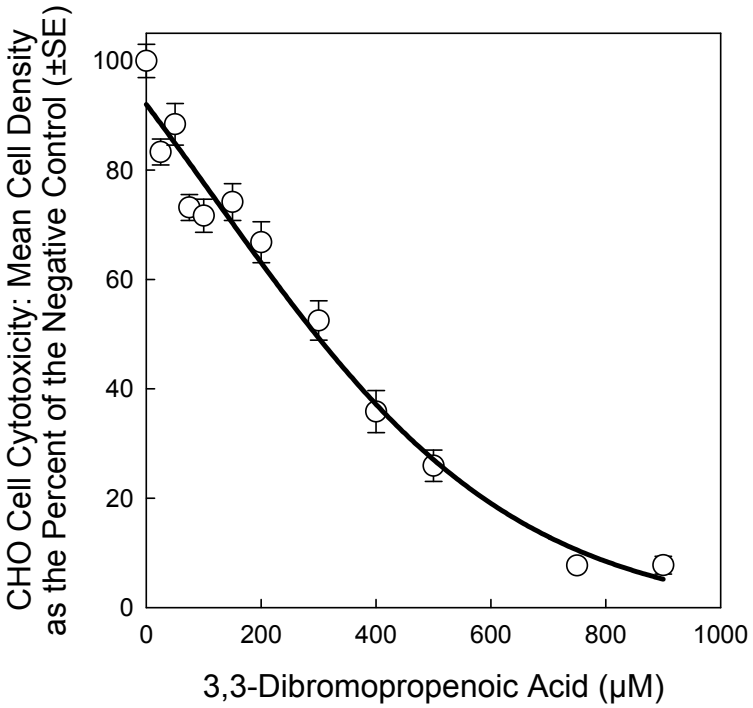


Figure 5.4 CHO cell chronic cytotoxicity concentration-response curve for 3,3-dibromopropenoic acid

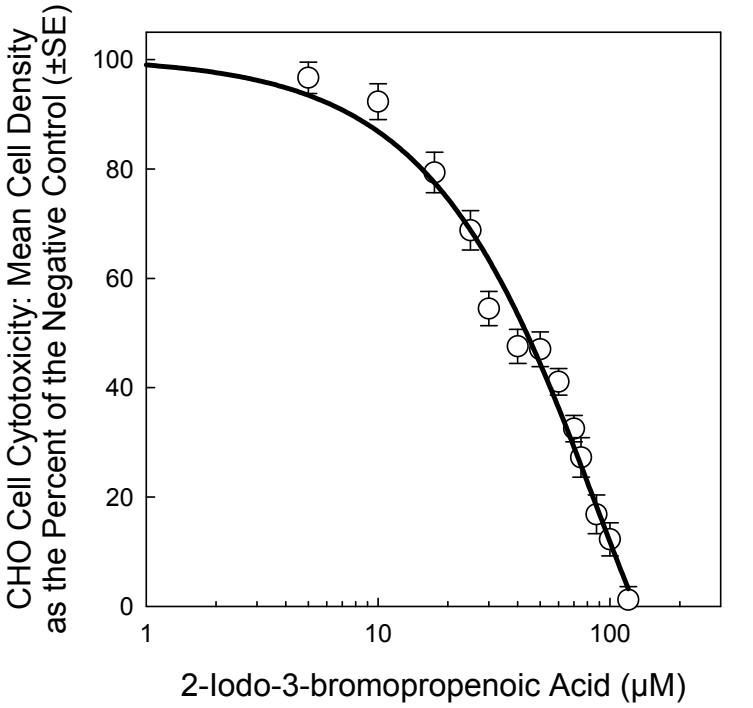


Figure 5.5 CHO cell chronic cytotoxicity concentration-response curve for 2-iodo-3-bromopropenoic acid

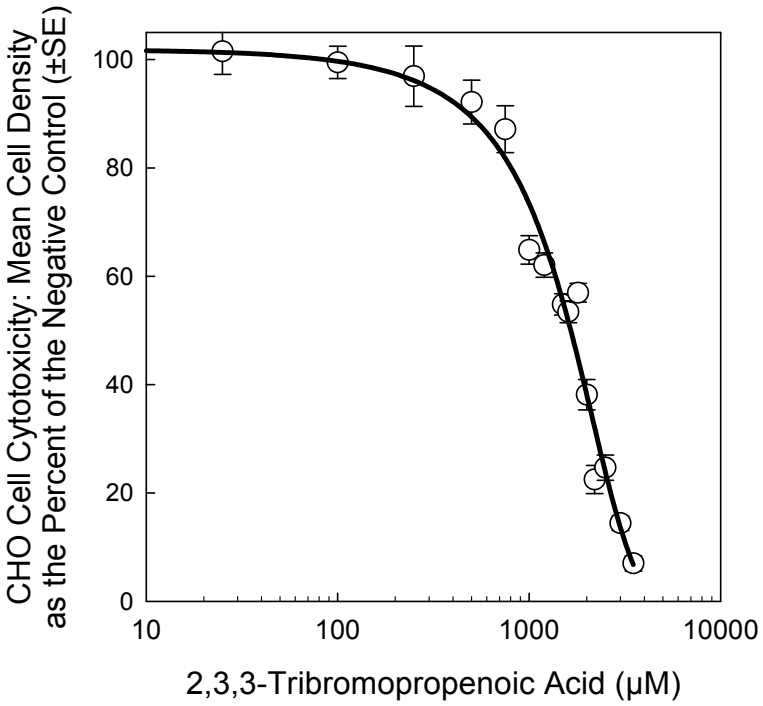


Figure 5.6 CHO cell chronic cytotoxicity concentration-response curve for 2,3,3-tribromopropenoic acid

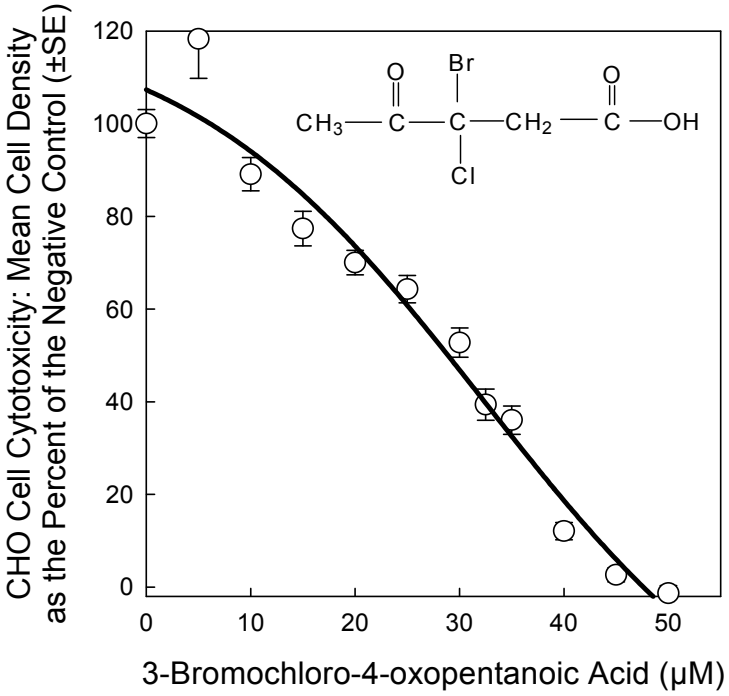


Figure 5.7 CHO cell chronic cytotoxicity concentration-response curve for 3-bromochloro-4-oxopentanoic acid

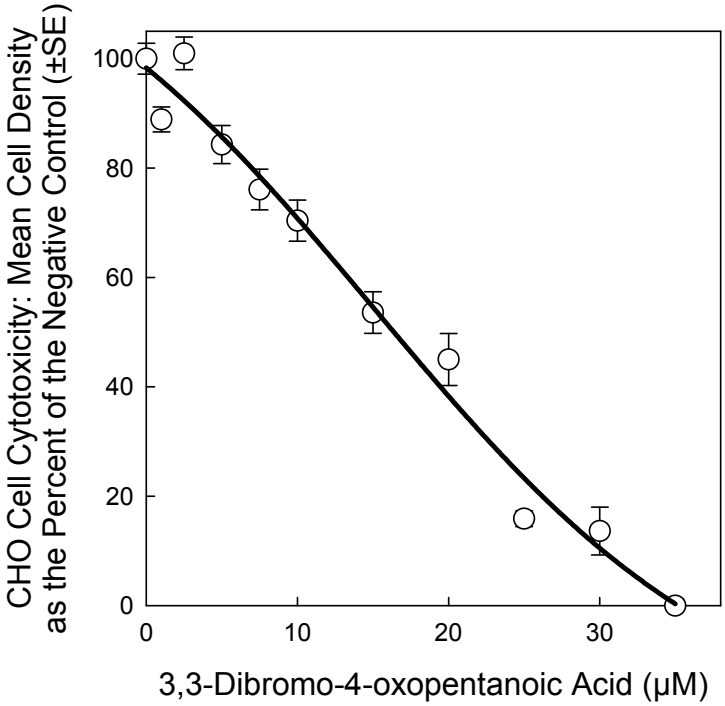


Figure 5.8 CHO cell chronic cytotoxicity concentration-response curve for 3,3-dibromo-4-oxopentanoic acid

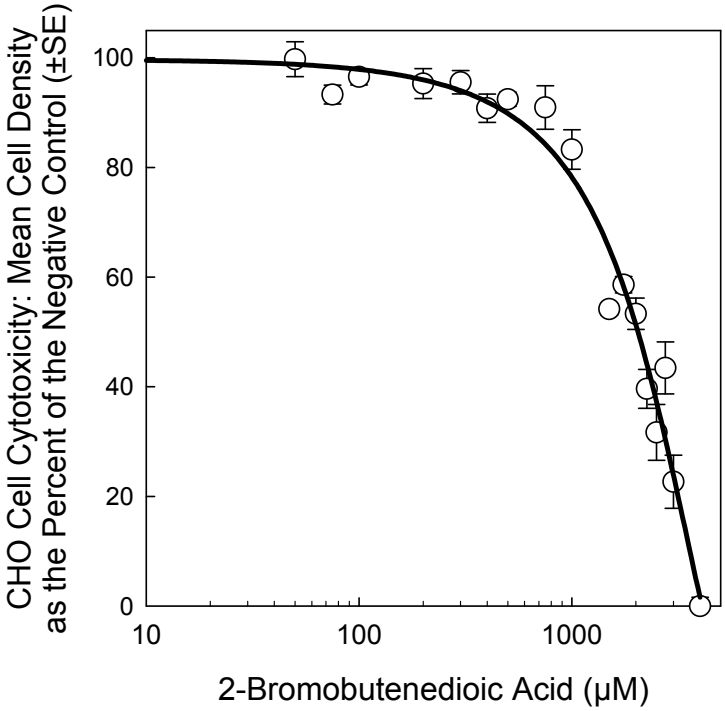


Figure 5.9 CHO cell chronic cytotoxicity concentration-response curve for 2-bromobutenedioic acid

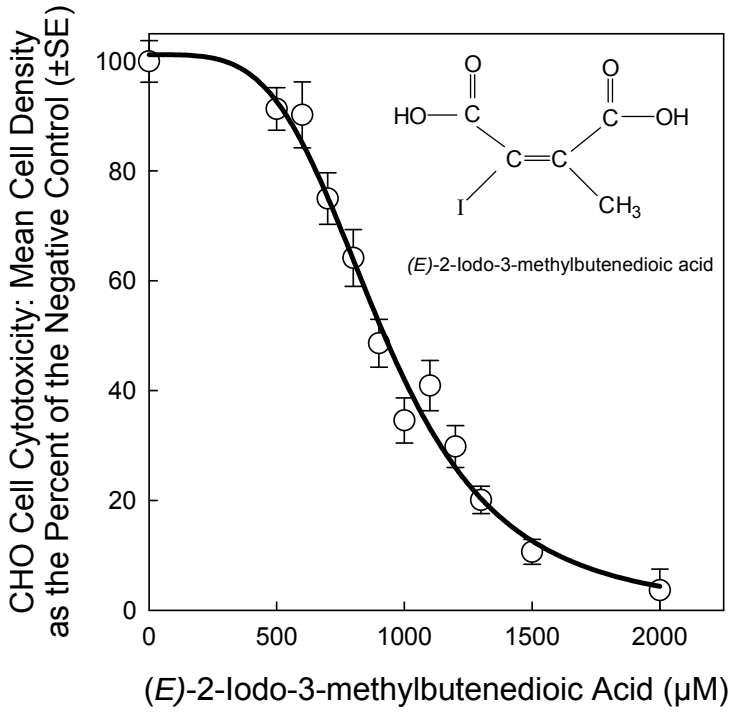


Figure 5.10 CHO cell chronic cytotoxicity concentration-response curve for (*E*)-2-iodo-3-methylbutenedioic acid

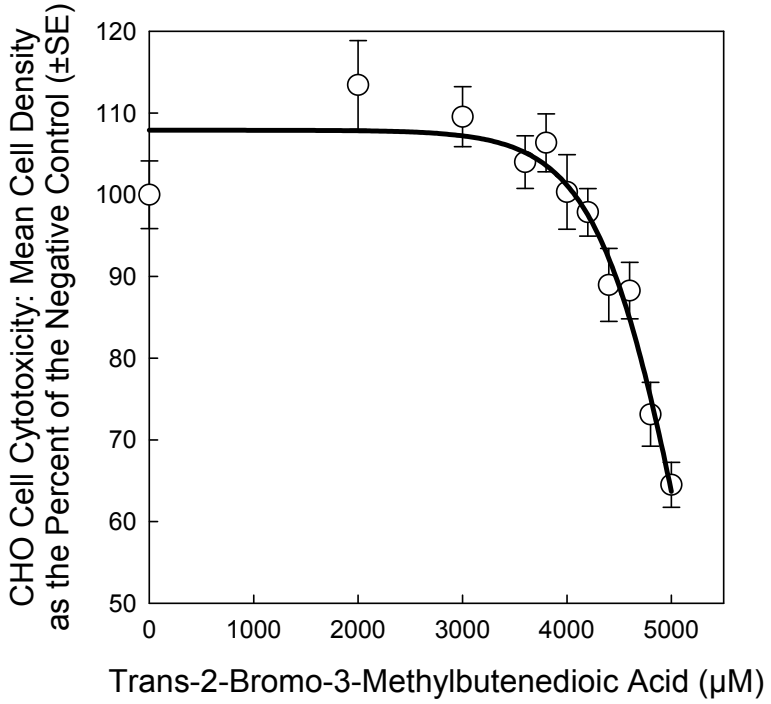


Figure 5.11 CHO cell chronic cytotoxicity concentration-response curve for 2-bromo-3-methylbutenedioic acid

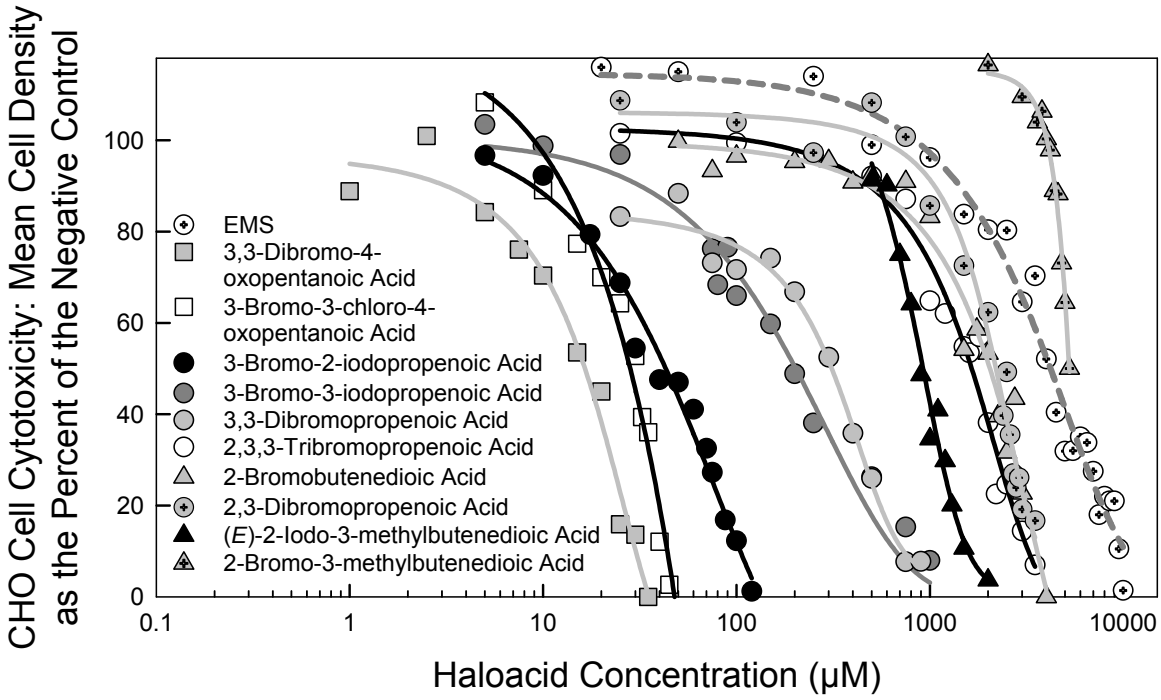


Figure 5.12 Comparison of the concentration-response curves for the CHO cell chronic cytotoxicity of the >2C-haloacids with EMS as the positive control

CHO CELL GENOTOXICITY ANALYSIS OF THE >2C-HALOACIDS

In this study ten >2C-haloacids were analyzed for their ability to induce genomic DNA damage in CHO cells; the results are presented in Table 5.2. In the table, the lowest concentration of a specific >2C-haloacid 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.

Table 5.2
CHO cell genotoxicity of the >2C-haloacid DBPs and related chemicals

Compound	Lowest Genotox. Conc. (M)	R^2	SCGE Gen. Potency (M)	ANOVA Test Statistic
3-Bromo-3-iodopropenoic acid	NS	–	NS	$F_{5,21} = 1.05$; $P = 0.42$
2,3-Dibromopropenoic acid	1.00×10^{-3}	0.97	7.85×10^{-3}	$F_{4,21} = 16.56$; $P \leq 0.01$
3,3-Dibromopropenoic acid	NS	–	NS	$F_{5,24} = 1.56$; $P = 0.21$
2-Iodo-3-bromopropenoic acid	7.50×10^{-3}	0.98	7.58×10^{-3}	$F_{9,28} = 8.42$; $P \leq 0.001$
2,3,3-Tribromopropenoic acid	NS	–	NS	$F_{5,22} = 1.75$; $P = 0.16$
3-Bromo-3-chloro-4-oxopentanoic acid	3.25×10^{-4}	0.97	3.58×10^{-4}	$F_{10,49} = 21.3$; $P \leq 0.001$
3,3-Dibromo-4-oxopentanoic acid	5.00×10^{-5}	0.98	9.03×10^{-5}	$F_{10,59} = 30.57$; $P \leq 0.001$
2-Bromobutenedioic acid	6.00×10^{-3}	0.99	5.90×10^{-3}	$F_{6,28} = 38.71$; $P \leq 0.001$
(*E*)-2-Iodo-3-methylbutenedioic acid	6.00×10^{-3}	0.98	6.00×10^{-3}	$F_{12,53} = 20.73$; $P \leq 0.001$
2-Bromo-3-methylbutenedioic acid	NS	–	NS	$F_{6,28} = 0.84$; $P = 0.55$

NS = not statistically different from the negative control.

The CHO cell concentration-response curves illustrating the induction of genomic DNA damage are presented for 3-bromo-3-iodopropenoic acid, 2,3-dibromopropenoic acid, 3,3-dibromopropenoic acid, 2-iodo-3-bromopropenoic acid, 2,3,3-tribromopropenoic acid, 3-bromo-3-chloro-4-oxopentanoic acid, 3,3-dibromo-4-oxopentanoic acid, 2-bromobutenedioic acid, (*E*)-2-iodo-3-methylbutenedioic acid, and *trans*-2-bromo-3-methylbutenedioic acid in Figures 5.13 – 5.22, respectively. A comparison of the CHO cell genotoxicity for these >2C-haloacids is presented in Figure 5.23.

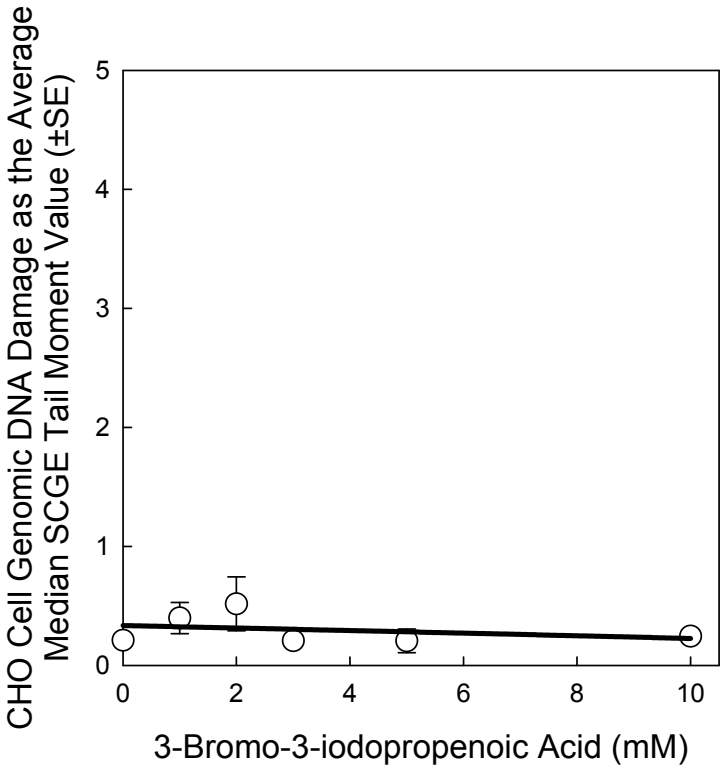


Figure 5.13 CHO cell SCGE genotoxicity concentration-response curve for 3-bromo-3-iodopropenoic acid

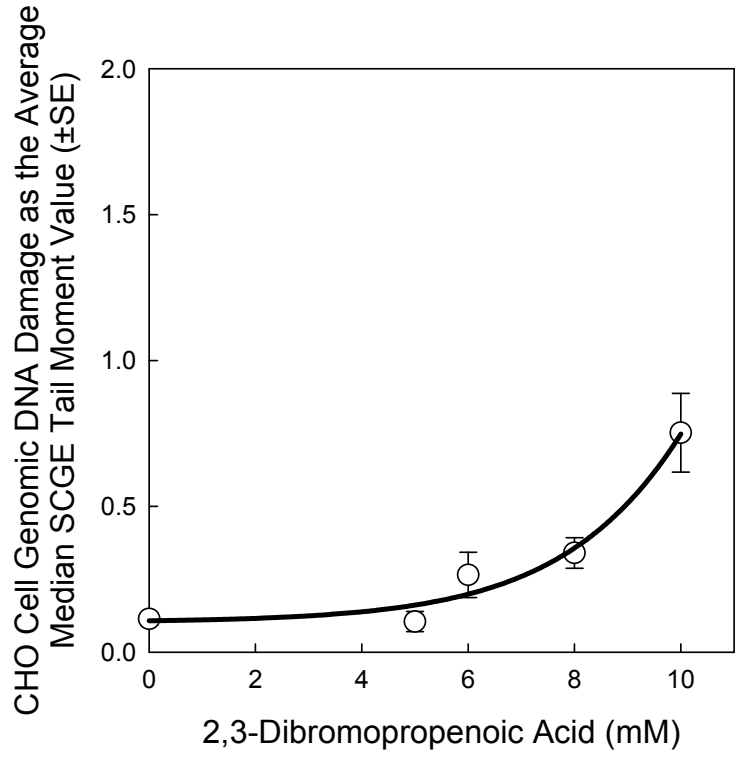


Figure 5.14 CHO cell SCGE genotoxicity concentration-response curve for 2,3-dibromopropenoic acid

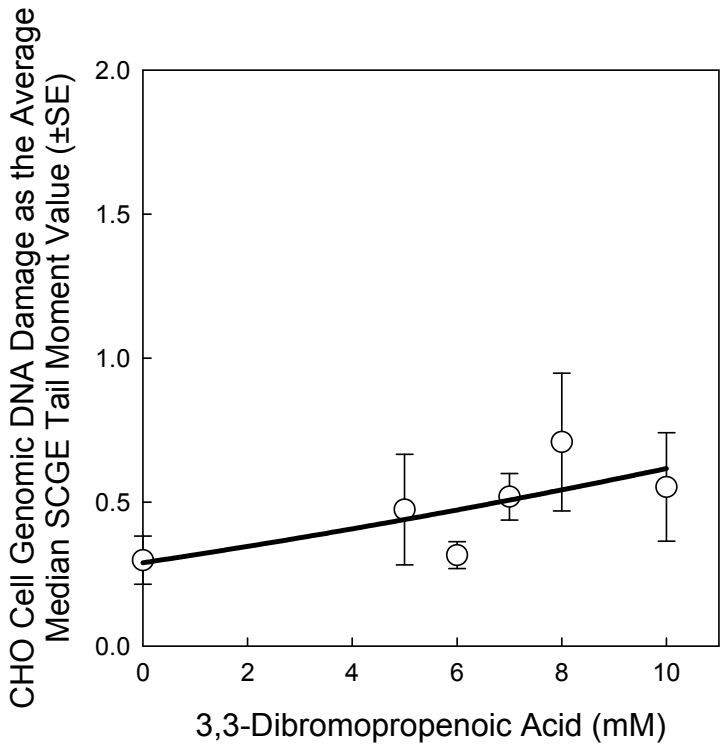


Figure 5.15 CHO cell SCGE genotoxicity concentration-response curve for 3,3-dibromopropenoic acid

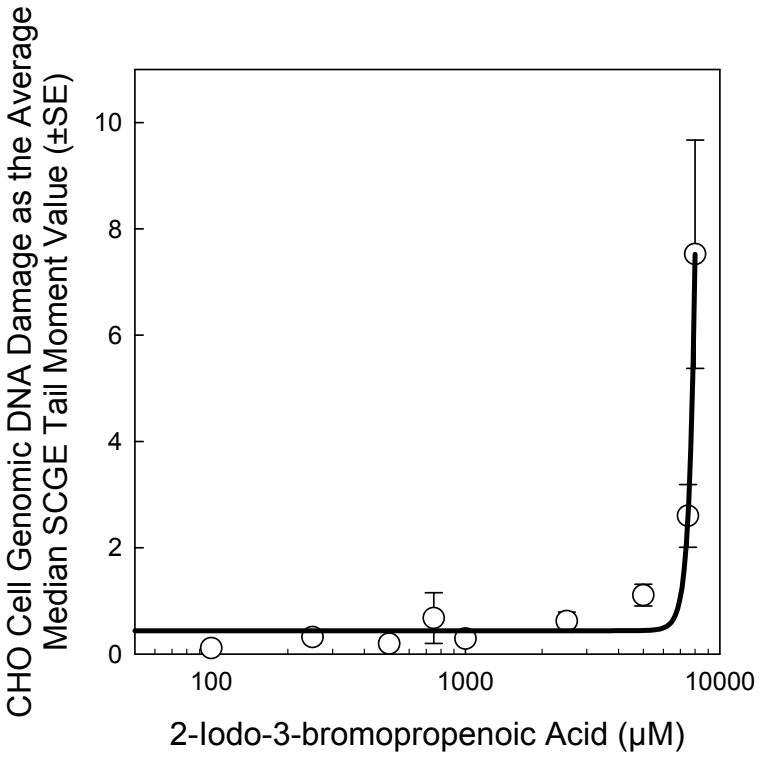


Figure 5.16 CHO cell SCGE genotoxicity concentration-response curve for 2-iodo-3-bromopropenoic acid

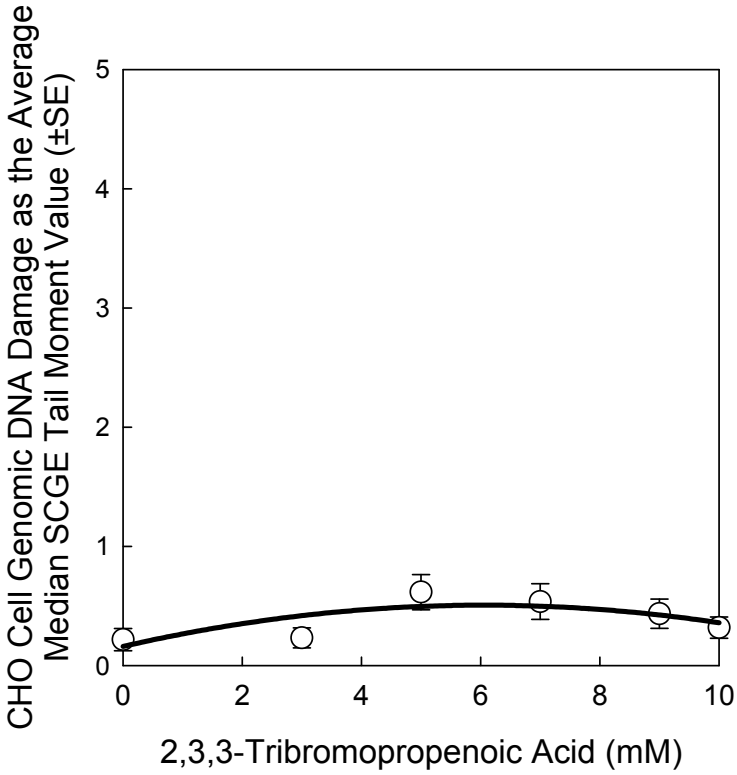


Figure 5.17 CHO cell SCGE genotoxicity concentration-response curve for 2,3,3-tribromopropenoic acid

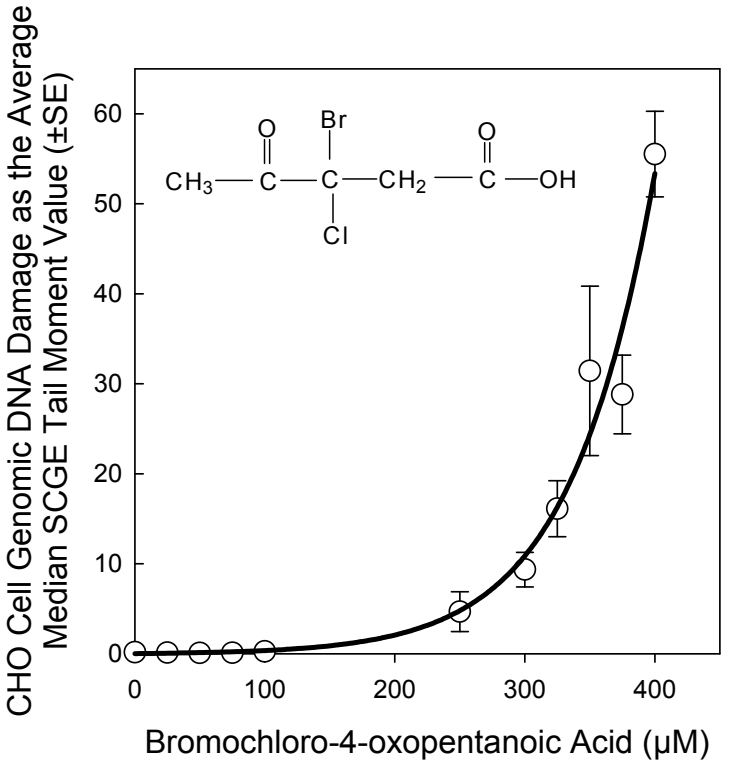


Figure 5.18 CHO cell SCGE genotoxicity concentration-response curve for 3-bromo-3-chloro-4-oxopentanoic acid

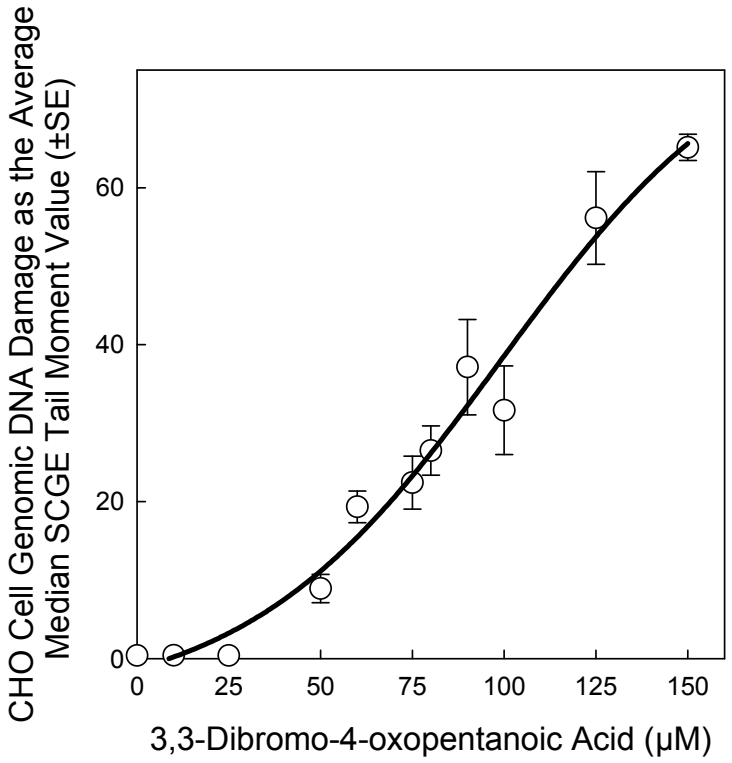


Figure 5.19 CHO cell SCGE genotoxicity concentration-response curve for 3,3-dibromo-4-oxopentanoic acid

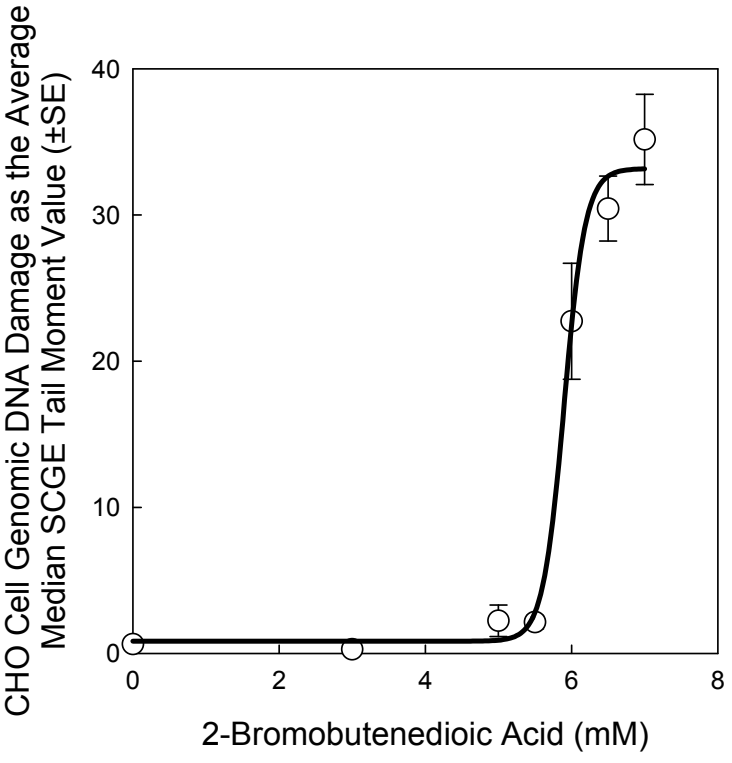


Figure 5.20 CHO cell SCGE genotoxicity concentration-response curve for 2-bromobutenedioic acid

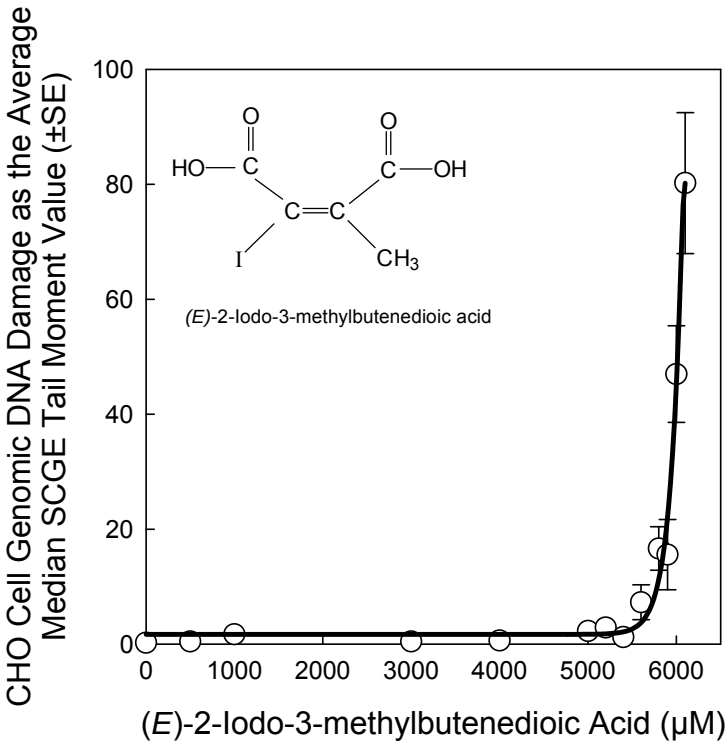


Figure 5.21 CHO cell SCGE genotoxicity concentration-response curve for (*E*)-2-iodo-3-methylbutenedioic acid

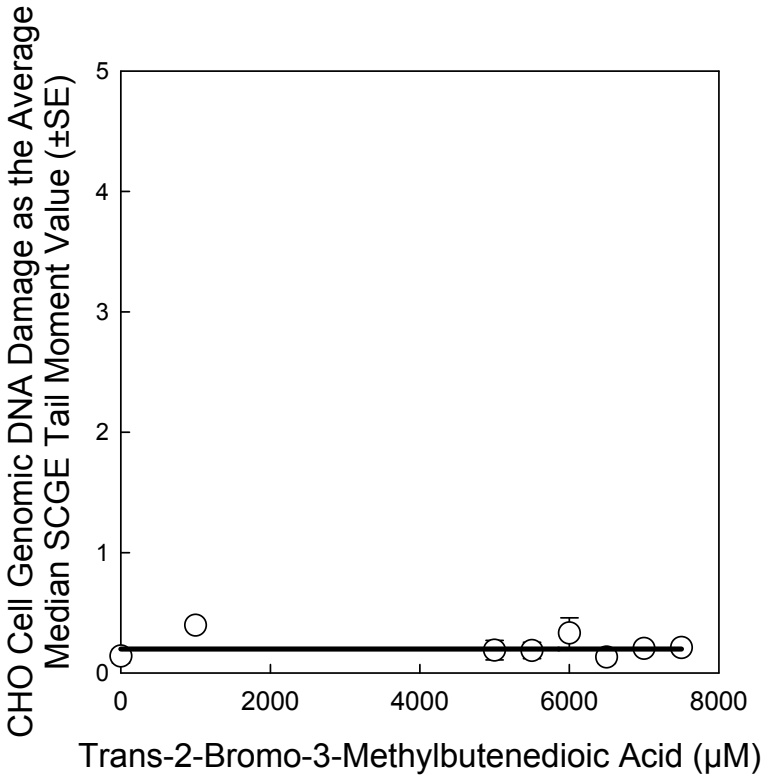


Figure 5.22 CHO cell SCGE genotoxicity concentration-response curve for trans-2-bromo-3-methylbutenedioic acid

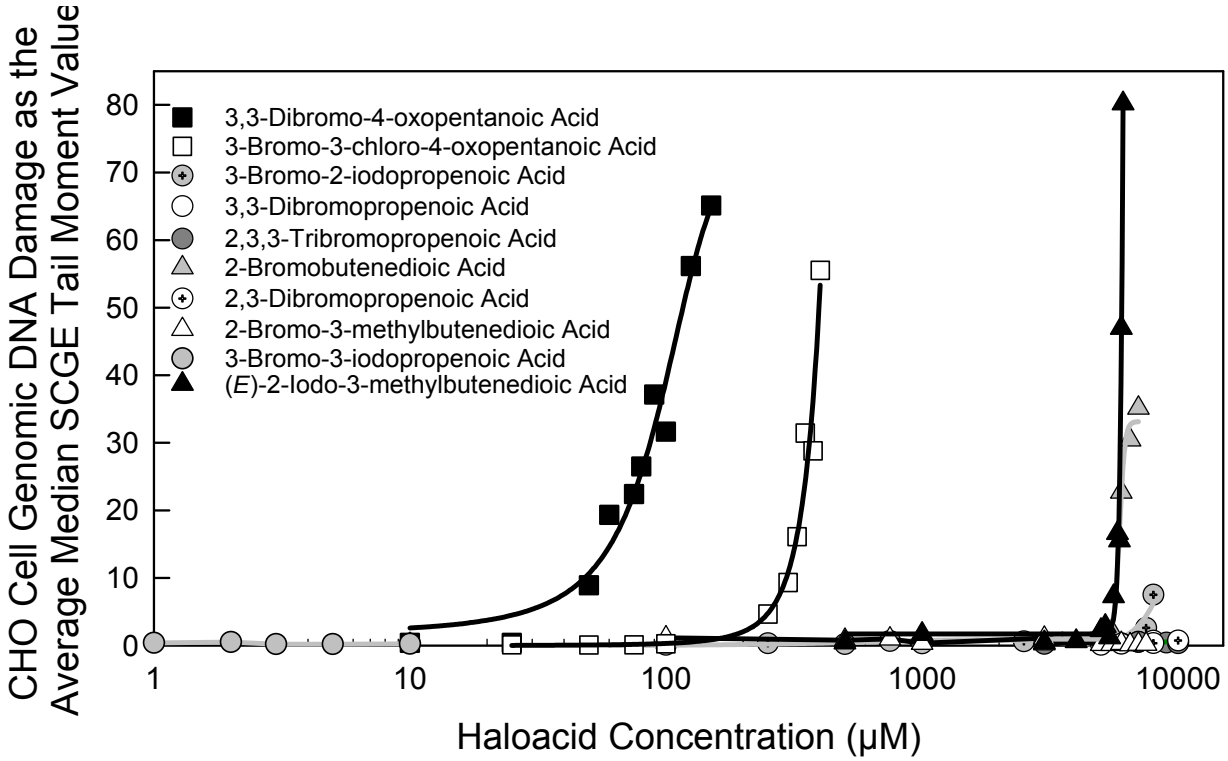


Figure 5.23 Comparison of the SCGE genotoxicity concentration-response curves of ten >2C-haloacids

COMPARATIVE CYTOTOXICITY AND GENOTOXICITY OF THE >2C-HALOACIDS

The >2C-haloacids were divided among the halopropenoic acids, halobutenedioic acids and the halooxopentanoic acids. A comparison of the relative CHO cell cytotoxicity and genotoxicity of the ten >2C-haloacids analyzed in this study is presented in Figures 5.12 and 5.23, respectively. Their rank order from high to low CHO cell chronic cytotoxicity, as defined by their %C½ values, is 3,3-dibromo-4-oxopentanoic acid > 3-bromo-3-chloro-4-oxopentanoic acid > 2-iodo-3-bromopropenoic acid > 3-bromo-3-iodopropenoic acid > 3,3-dibromopropenoic acid > (*E*)-2-iodo-3-methylbutenedioic acid > 2,3,3-tribromopropenoic acid > 2-bromobutenedioic acid > 2,3-dibromopropenoic acid > 2-bromo-3-methylbutenedioic acid (Table 5.1). The rank order from high to low CHO cell genotoxicity, based on their SCGE Genotoxic Potency values, is 3,3-dibromo-4-oxopentanoic acid > 3-bromo-3-chloro-4-oxopentanoic acid > 2-bromobutenedioic acid > (*E*)-2-iodo-3-methylbutenedioic acid > 2-iodo-3-bromopropenoic acid > 2,3-dibromopropenoic acid. In this assay 3-bromo-3-iodopropenoic acid, 3,3-dibromopropenoic acid, 2,3,3-tribromopropenoic acid, and 2-bromo-3-methylbutenedioic acid were refractory (Table 5.2). As a chemical class the >2C-haloacids expressed CHO cell cytotoxicity similar to the haloacetic acids, however, they express lower genotoxic effects (see Chapter 9).

Although there is a trend toward higher cytotoxicity and genotoxicity with increased number of carbon atoms of the DBP, the functional groups also have a profound effect on the bi-

ological impact of the chemical. The effect of the halogen species and the oxo-functional group is observed with 3,3-dibromo-4-oxopentanoic acid approximately 1.8× more cytotoxic and 4× more genotoxic than 3-bromo-3-chloro-4-oxopentanoic acid (Tables 5.1 and 5.2). The consequence of the halogen species and functional groups is evident when comparing these agents with another 4-oxo-DBP. The halogenated furanone, MX has two open-ring tautomeric forms at physiological pH: (*Z*)-2-chloro-3-dichloromethyl-4-oxobutenoic acid and (*E*)-2-chloro-3-dichloromethyl-4-oxobutenoic acid. Although the brominated and bromo-chloro analogues of this DBP were not evaluated in this study, 3,3-dibromo-4-oxopentanoic acid was nearly 17× more cytotoxic and approximately 5× more genotoxic than MX (Tables 5.1 and 5.2) (Plewa et al. 2002). When the comparison was between 3-bromo-3-chloro-4-oxopentanoic acid and MX, the oxopentanoic acid was approximately 10× more cytotoxic and about 70% as genotoxic in CHO cells (Tables 5.1 and 5.2) (Plewa et al. 2002). There was a slight trend of >2C-haloacids with iodine and bromine possessing more cytotoxicity and genotoxicity than their brominated only analogues. A comparison of 2-iodo-3-methylbutenedioic acid with 2-bromo-3-methylbutenedioic acid revealed that the former was approximately 6× more cytotoxic. In addition 2-iodo-3-methylbutenedioic acid was genotoxic in CHO cells while the brominated analogue was refractory.

CHAPTER 6
HALOACETONITRILES

INTRODUCTION

Seven haloacetonitriles were evaluated in this study, bromoacetonitrile, bromochloroacetonitrile, chloroacetonitrile, dibromoacetonitrile, dichloroacetonitrile, iodoacetonitrile, and trichloroacetonitrile (Table 2.1)

OCCURRENCE

The haloacetonitriles were measured in several occurrence studies (Krasner et al. 2006; Weinberg et al. 2002; Krasner et al. 1989; Williams, LeBel, and Benoit 1997; McGuire, McLain, and Obolensky 2002) with bromochloroacetonitrile, chloroacetonitrile, dibromoacetonitrile, and trichloroacetonitrile (HAN4) the most commonly measured species. In the U.S. EPA's Information Collection Rule (ICR), the haloacetonitriles (HAN4) ranged from <0.5 to 41.0 µg/L, and were generally 12% of the levels of the four regulated trihalomethanes. These haloacetonitriles were formed using chlorine and/or chloramine disinfection; plants using chloramines (with and without chlorine) had the highest levels. Higher haloacetonitrile levels were from distribution system waters treated with post-chloramination versus free chlorine. However, the increased haloacetonitrile levels with chloramination may be a result of higher total organic carbon (TOC) levels in their source waters (McGuire, McLain, and Obolensky 2002). Haloacetonitriles were frequently found in Canadian drinking waters with dichloroacetonitrile in 97% of all samples (Williams, LeBel, and Benoit 1997). Although the haloacetonitriles are not regulated in the United States, the World Health Organization published a guideline of 70 µg/L for dibromoacetonitrile and a provisional guideline of 20 µg/L for dichloroacetonitrile (World Health Organization 2006).

Several other haloacetonitriles were detected in a recent nationwide DBP occurrence study (Krasner et al. 2006). These included bromo-, bromodichloro-, dibromochloro-, and tribromoacetonitrile plus the four ICR (HAN4) listed above. Total haloacetonitrile levels reached a maximum of 14 µg/L and were approximately 10% of the THM4 levels, although a maximum of 25% was observed. When bromide was present in the source waters, more brominated species were formed. This shift was observed with high bromide waters in Israel (Richardson et al. 2003), where chlorine dioxide disinfection formed dibromoacetonitrile, as well as a new bromonitrile species (3-bromopropanenitrile).

GENOTOXICITY

Dichloroacetonitrile, bromochloroacetonitrile, chloroacetonitrile, and trichloroacetonitrile were mutagenic in *Salmonella typhimurium* (Bull et al. 1985; Muller-Pillet et al. 2000). Haloacetonitriles directly induced sister chromatid exchanges (SCE) in CHO cells (Bull et al. 1985). The rank order of the direct acting genotoxic activity was dibromoacetonitrile > bromochloroacetonitrile > trichloroacetonitrile > dichloroacetonitrile > chloroacetonitrile, where brominated and di- and tri-halogenated haloacetonitriles were more toxic. Haloacetonitriles produced DNA strand breaks in human lymphoblastic cells, with trichloroacetonitrile the most potent (Daniel et al.

1986; Lin et al. 1986). Although haloacetonitriles possessed direct acting alkylating activity, there was no correlation between DNA alkylation potential and their ability to produce DNA strand breaks (Lin et al. 1986). Using the SCGE assay with HeLa S3 cells, chloroacetonitrile, bromoacetonitrile, dichloroacetonitrile, dibromoacetonitrile, and trichloroacetonitrile were positive; the more halogenated haloacetonitriles and brominated haloacetonitriles caused greater amounts of damage (Muller-Pillet et al. 2000). Haloacetonitriles directly interacted with calf thymus DNA in the order of bromoacetonitrile > chloroacetonitrile > dichloroacetonitrile > trichloroacetonitrile. A DNA adduct was identified as 7-(cyanomethyl) guanine (Nouraldeen and Ahmed 1996) and haloacetonitriles bound to a nucleophilic trapping agent and formed a covalent bond to polyadenylic acid (Daniel et al. 1986).

In vivo studies in rats demonstrated differential metabolism and excretion with a rank order of chloroacetonitrile > bromochloroacetonitrile > dichloroacetonitrile > dibromoacetonitrile > > trichloroacetonitrile (Lin et al. 1986). The haloacetonitriles initiated skin tumors in mice with a rank order of dibromoacetonitrile > bromochloroacetonitrile > chloroacetonitrile (Bull et al. 1985).

CHO CELL CYTOTOXICITY ANALYSIS OF THE HALONITRILES

The CHO cell chronic cytotoxicity of the seven halonitriles analyzed in this study are presented in Table 6.1. 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.

Table 6.1
CHO cell chronic cytotoxicity of the haloacetonitrile DBPs and related chemicals

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}	$F_{17,282} = 36.8$; $P \leq 0.001$

The CHO cell chronic cytotoxicity concentration-response curves are presented for each halonitrile in the following figures: bromoacetonitrile (Figure 6.1), bromochloroacetonitrile (Figure 6.2), chloroacetonitrile (Figure 6.3), dibromoacetonitrile (Figure 6.4), dichloroacetonitrile (Figure 6.5), iodoacetonitrile (Figure 6.6), and trichloroacetonitrile (Figure 6.7). A comparison of the relative cytotoxicity of the halonitriles analyzed in this study is presented in Figure 6.8.

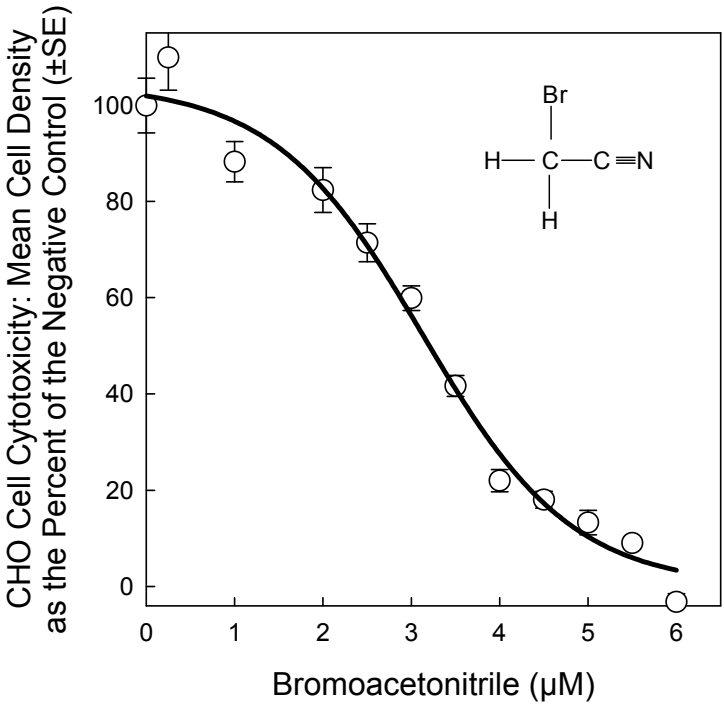


Figure 6.1 CHO cell chronic cytotoxicity concentration-response curve for bromoacetonitrile

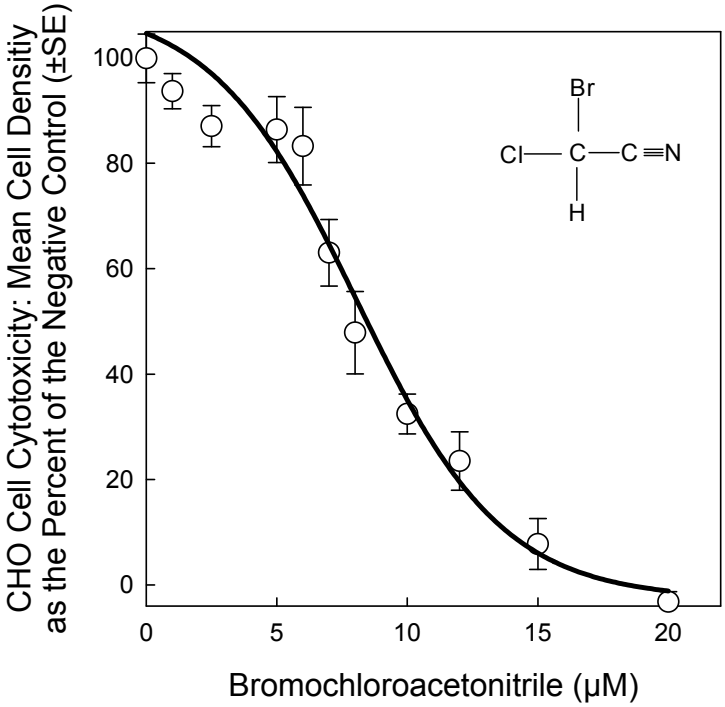


Figure 6.2 CHO cell chronic cytotoxicity concentration-response curve for bromochloroacetonitrile

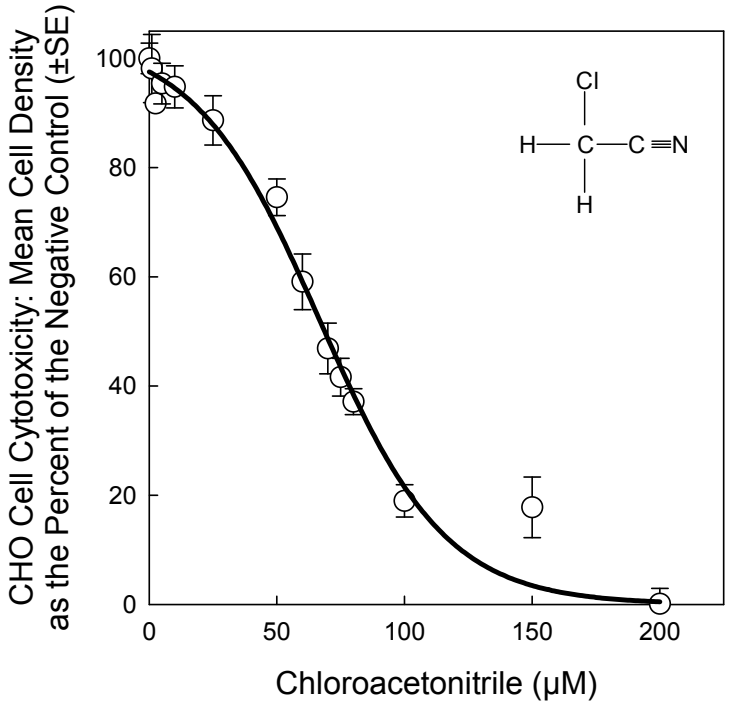


Figure 6.3 CHO cell chronic cytotoxicity concentration-response curve for chloroacetonitrile

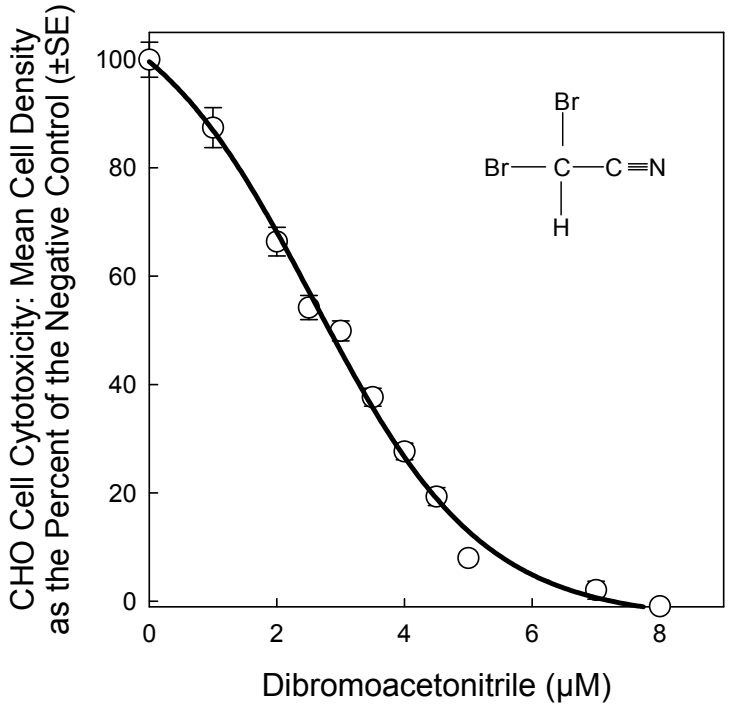


Figure 6.4 CHO cell chronic cytotoxicity concentration-response curve for dibromoacetonitrile

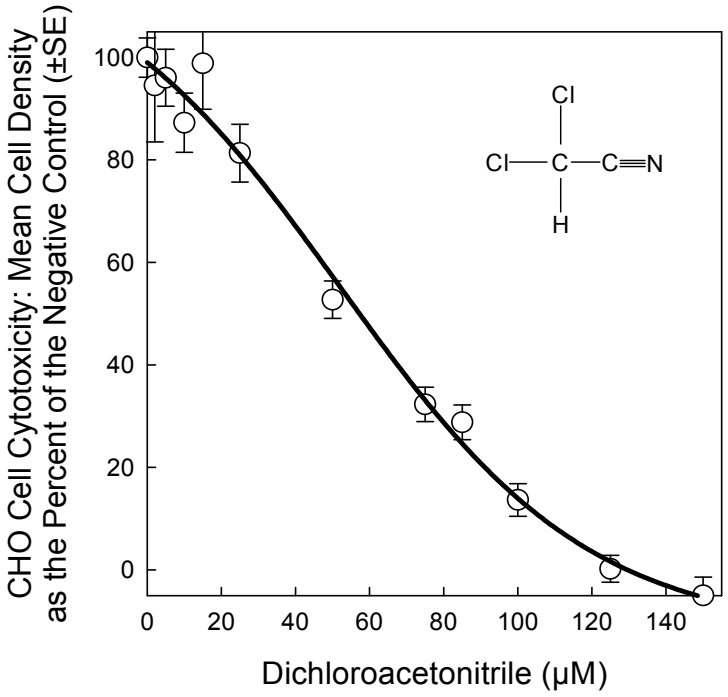


Figure 6.5 CHO cell chronic cytotoxicity concentration-response curve for dichloroacetonitrile

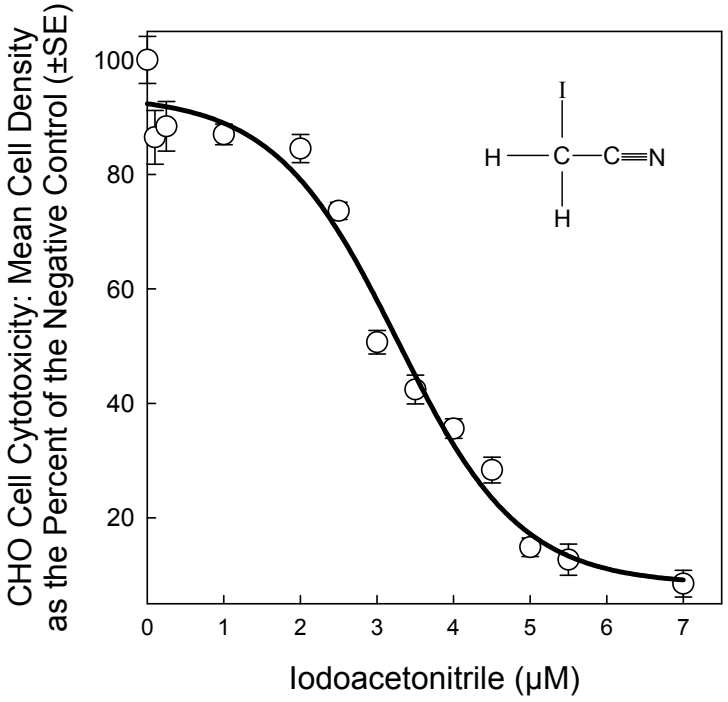


Figure 6.6 CHO cell chronic cytotoxicity concentration-response curve for iodoacetonitrile

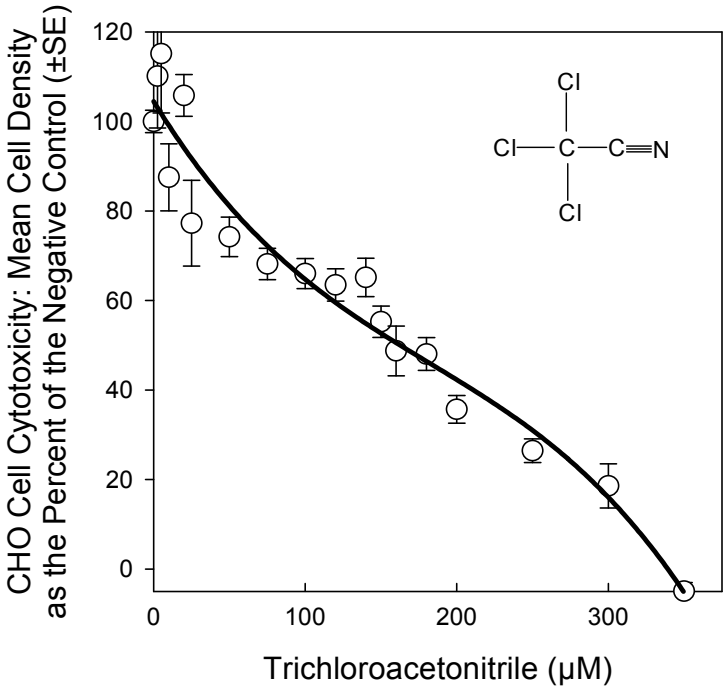


Figure 6.7 CHO cell chronic cytotoxicity concentration-response curve for trichloroacetonitrile

CHO CELL GENOTOXICITY ANALYSIS OF THE HALONITRILES

In this study seven halonitriles were analyzed for their ability to induce genomic DNA damage in CHO cells; the results are presented in Table 6.2. In the table, 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.

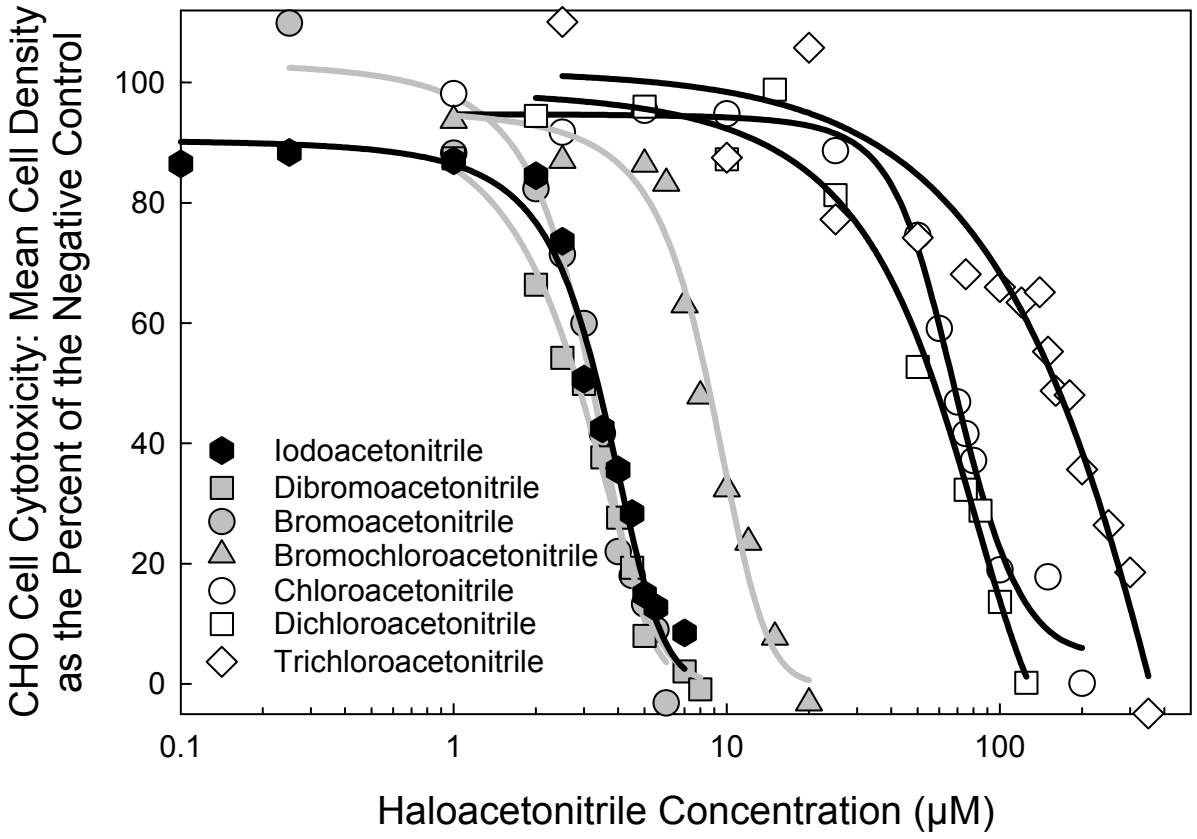


Figure 6.8 Comparison of the concentration-response curves for the CHO cell chronic cytotoxicity of seven haloacetonitriles

Table 6.2
CHO cell genotoxicity of the haloacetonitrile DBPs and related chemicals

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}	$F_{7, 32} = 30.5$; $P \leq 0.001$

The CHO cell concentration-response curves illustrating the induction of genomic DNA damage are presented for bromoacetonitrile, bromochloroacetonitrile, chloroacetonitrile, dibromoacetonitrile, dichloroacetonitrile, iodoacetonitrile, and trichloroacetonitrile in Figures 6.9 –

6.15, respectively. A comparison of the CHO cell genotoxicity for these halonitriles is presented in Figure 6.16.

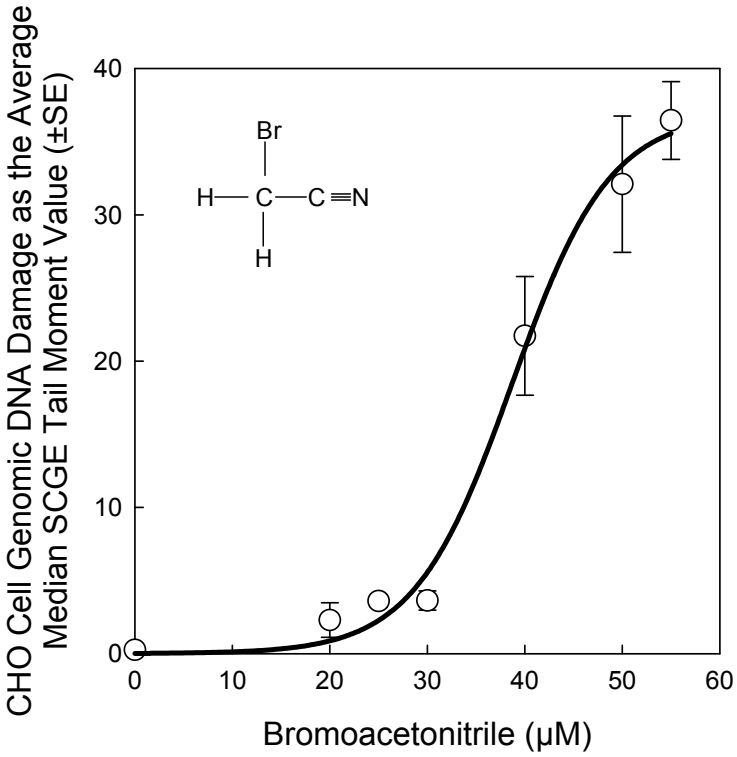


Figure 6.9 CHO cell SCGE genotoxicity concentration-response curve for bromoacetonitrile

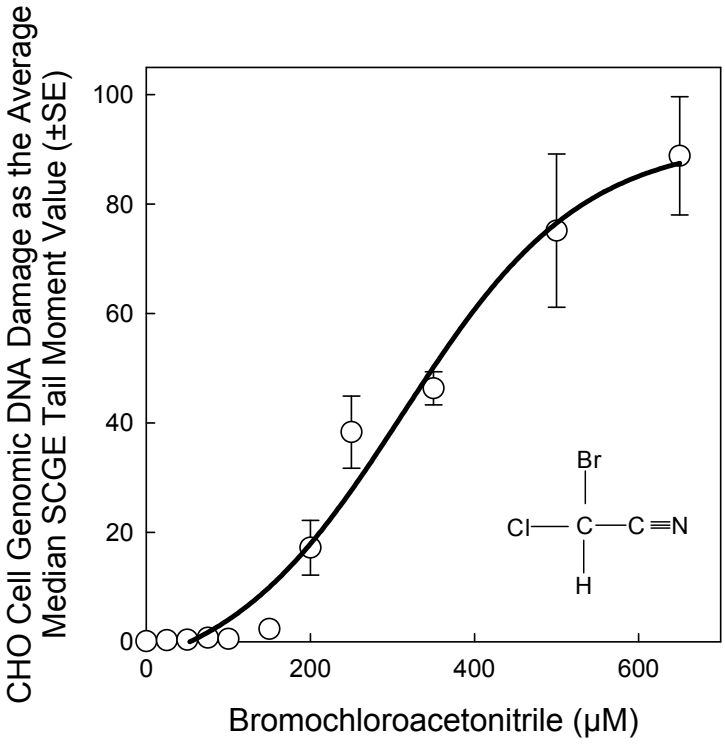


Figure 6.10 CHO cell SCGE genotoxicity concentration-response curve for bromochloroacetonitrile

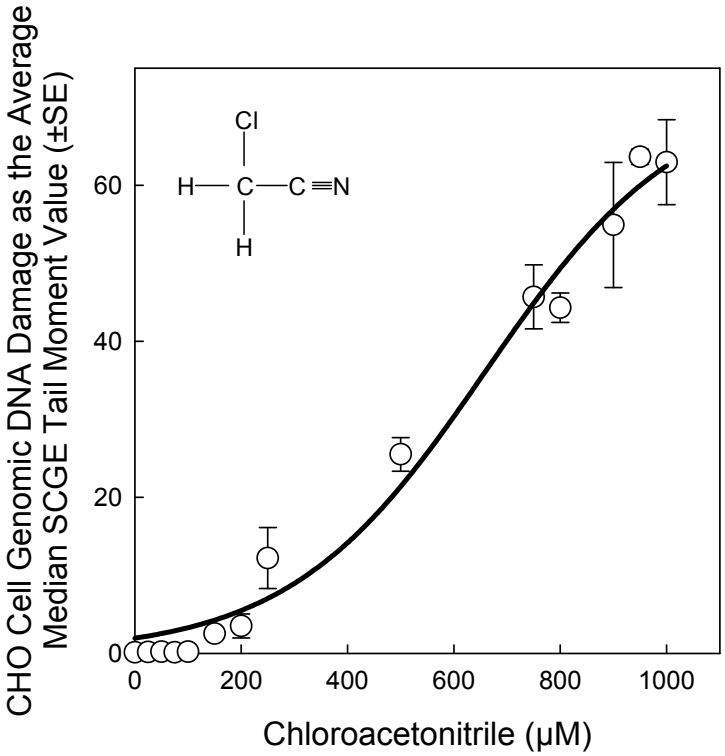


Figure 6.11 CHO cell SCGE genotoxicity concentration-response curve for chloroacetonitrile

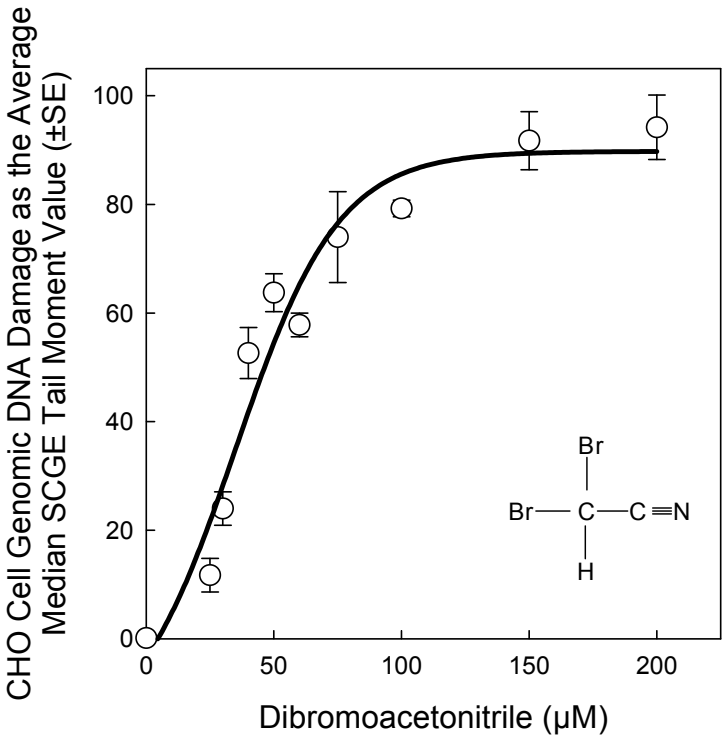


Figure 6.12 CHO cell SCGE genotoxicity concentration-response curve for dibromoacetonitrile

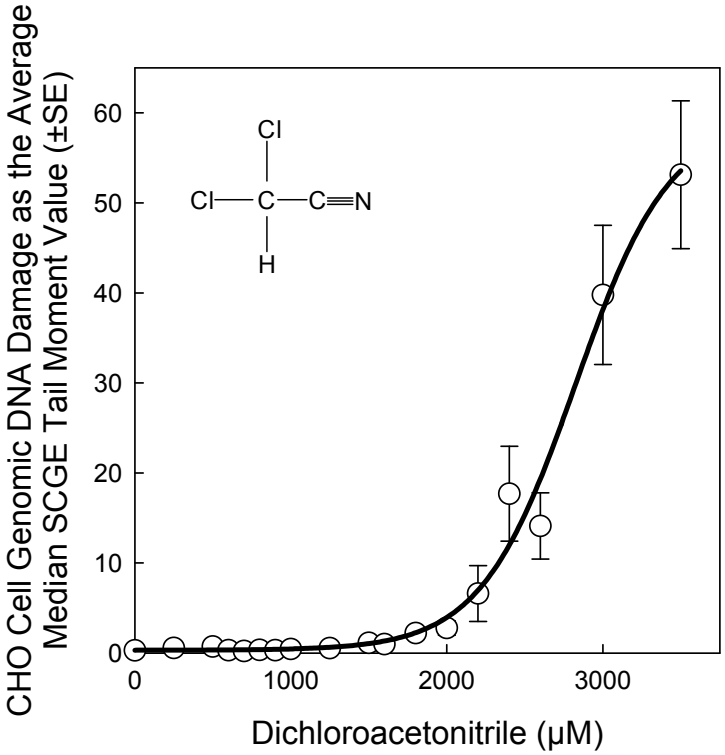


Figure 6.13 CHO cell SCGE genotoxicity concentration-response curve for dichloroacetonitrile

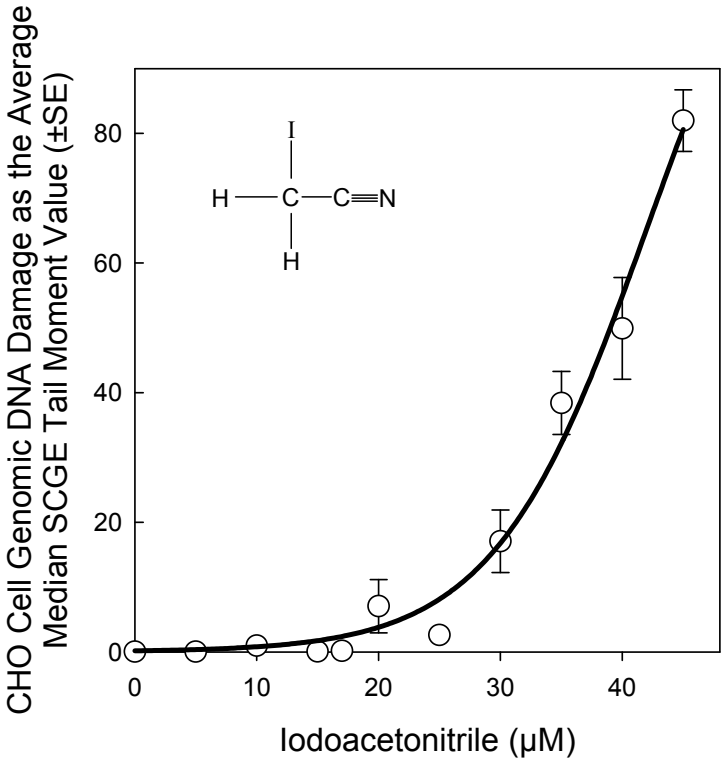


Figure 6.14 CHO cell SCGE genotoxicity concentration-response curve for iodoacetonitrile

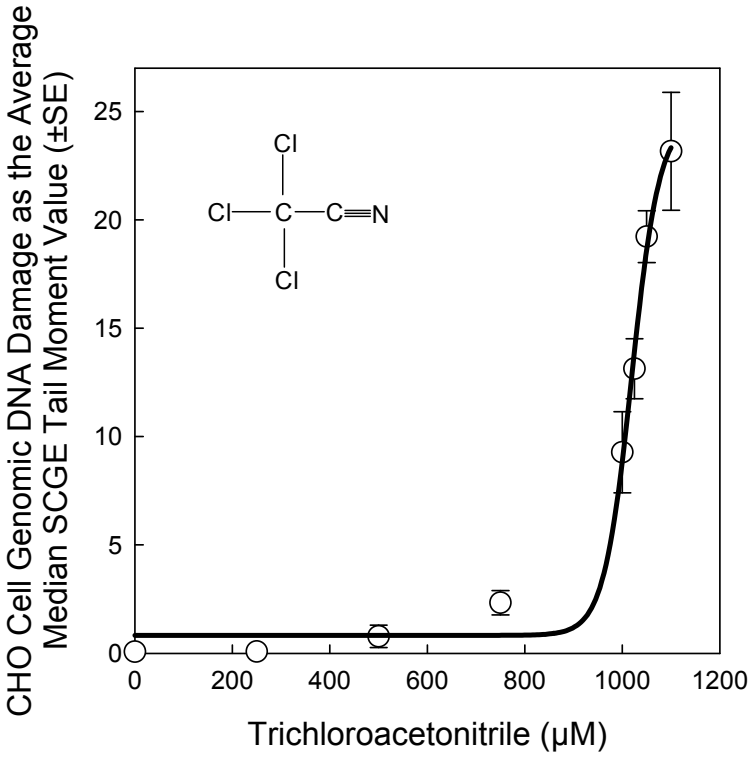


Figure 6.15 CHO cell SCGE genotoxicity concentration-response curve for trichloroacetonitrile

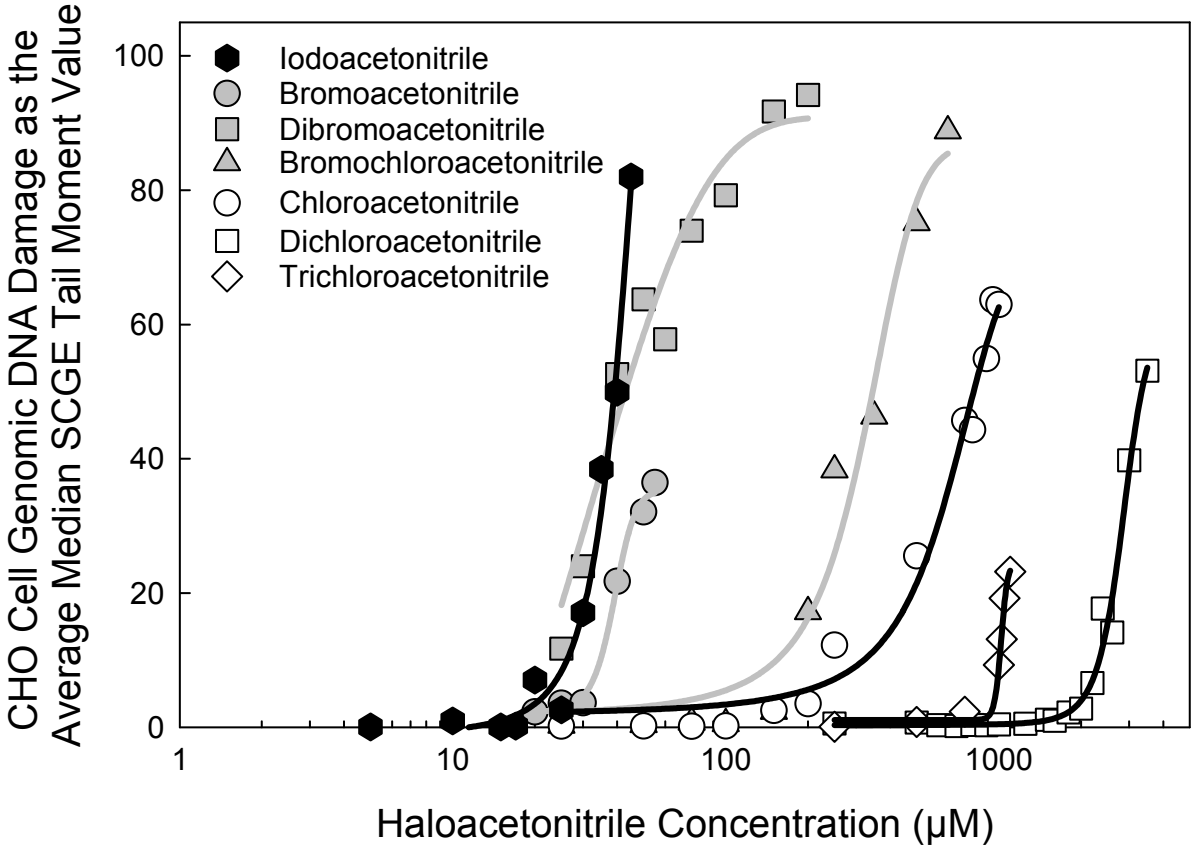


Figure 6.16 Comparison of the SCGE genotoxicity concentration-response curves of seven haloacetonitriles

COMPARATIVE CYTOTOXICITY AND GENOTOXICITY OF THE HALOACETONITRILES

Comparison of the Relative Chemical and Biological Activities of the Haloacetonitriles

A number of comparative haloacetonitrile studies are summarized in Table 6.3. The data were normalized to the response expressed by chloroacetonitrile. Using a Pearson Product Moment Multiple Correlation statistic, the relative activities of 6 haloacetonitriles for metabolism, alkylation potential, DNA strand breaks, genotoxicity, and toxicity were compared in a number of assays with the CHO cell cytotoxicity and genotoxicity data of the present study (Table 6.3). The endpoints included inhibition of dimethylnitrosamine-demethylase (DMN-DM) (Lin et al. 1986), the extent metabolized in rats (Pereira, Lin, and Mattox 1984), alkylation potential (Daniel et al. 1986), induction of DNA strand breaks (Daniel et al. 1986), genotoxicity in the *Escherichia coli* SOS chromotest (Le Curieux et al. 1995), clastogenicity in newt larvae (Le Curieux et al. 1995), sister chromatid exchange (SCE) in CHO cells (Bull et al. 1985), DNA damage in HeLa cells (Muller-Pillet et al. 2000), skin tumor induction (Bull et al. 1985), toxicity in *E. coli* and *Pleurodeles* newt (Le Curieux et al. 1995), and, finally, the CHO chronic cytotoxicity and genomic DNA damage reported here. No significant correlation was observed among the meta-

bolism of 5 haloacetonitriles (Daniel et al. 1986) and chronic cytotoxicity or genomic DNA damage in CHO cells. The *in vitro* inhibition of rodent microsomal DMN-DM was highly correlated with the alkylation potential, *E. coli* SOS genotoxicity, enhanced somatic chromosomal recombination (SCE) in CHO cells, genomic DNA damage in HeLa cells, and CHO cell chronic cytotoxicity ($r = 0.88$, $P < 0.05$; $r = 0.91$, $P < 0.03$; $r = 0.93$, $P < 0.02$; $r = 0.99$, $P < 0.002$; and $r = 0.91$, $P < 0.03$, respectively). Haloacetonitrile alkylation potential was highly correlated with measurements of mammalian cell genotoxicity: CHO SCE ($r = 0.98$, $P < 0.004$), HeLa cell SCGE ($r = 0.97$, $P < 0.03$), and CHO cell SCGE ($r = 0.97$, $P < 0.006$), as well as CHO cell cytotoxicity ($r = 0.99$, $P < 0.001$). Interestingly, DNA strand breakage using the alkaline unwinding procedure (Daniel et al. 1986) did not demonstrate significant correlations with other measurements of DNA damage. However, a high correlation was observed between CHO cell SCGE and HeLa cell SCGE ($r = 0.99$, $P < 0.01$), CHO cell SCE and CHO cell SCGE ($r = 0.96$, $P < 0.01$). and HeLa cell SCGE and CHO cell SCE ($r = 0.99$, $P < 0.004$). The induction of SOS *E. coli* toxicity and newt larva toxicity were highly related ($r = 0.98$, $P < 0.001$), as was the induction of chromosome breaks in the newt and genomic DNA damage in CHO cells ($r = 0.87$, $P < 0.03$). The data presented in this study expressed a significant and high correlation with other toxicity endpoints (Table 6.3). CHO cell chronic cytotoxicity was highly correlated with DMN-DM inhibition ($r = 0.91$, $P < 0.03$), alkylation potential ($r = 0.99$, $P < 0.001$), CHO cell SCE ($r = 0.99$, $P < 0.001$), HeLa cell SCGE ($r = 0.99$, $P < 0.006$), and CHO cell SCGE ($r = 0.96$, $P < 0.003$). CHO cell SCGE were also highly correlated with alkylation potential ($r = 0.97$, $P < 0.006$), CHO SCE ($r = 0.96$, $P < 0.001$), HeLa cell SCGE ($r = 0.99$, $P < 0.01$), and newt micronucleus induction ($r = 0.87$, $P < 0.03$). The induction of skin tumors expressed an association with alkylation potential, SCE induction, and induced genomic DNA damage in HeLa cells and CHO cells, as well as chronic cytotoxicity in CHO cells. However, this modest association ($r = 0.81$ to 0.74) was not statistically significant.

Structure-Activity Relationships (SAR) and Factors Affecting the Toxicity of Haloacetonitriles

The structure activity relationships (SAR) of the haloacetonitriles are interesting, but somewhat complicated. Haloacetonitriles have two potential electrophilic reactive centers: (i) displacement of a halogen atom at the α carbon by S_N2 reaction, and (ii) addition at the partially positively charged carbon of the cyano group (Lin and Guion 1989). Both reactions could contribute to the cytotoxicity or genotoxicity of the haloacetonitriles.

The S_N2 reactivity of the haloacetonitriles is dependent on the leaving tendency of the halogen and the degree of halogenation. The S_N2 reactivity of an alkyl iodide is 3-5× greater than that of alkyl bromide which is 50× greater than alkyl chloride (Loudon 1995); a similar relative order is expected for monohaloacetonitriles. The leaving tendency of a halogen is expected to decrease with increasing halogenation; therefore, the alkylating potential of the haloacetonitriles is also expected to decrease. Both the alkylating potential (Daniel et al. 1986) and DNA interaction with calf thymus DNA (Nouraldeen and Ahmed 1996) are consistent with the SAR expectation. The ability of the haloacetonitriles to bind to DNA may be significantly affected by the presence of glutathione (GSH).

Table 6.3
Comparison of the Relative Chemical and Biological Activities of the Haloacetonitriles [a]

HAN [b]	Inhib. DMN DM [c]	Extent Met. [d]	Alk. Pot [e]	DNA Strand Break. [f]	SOS GT [g]	Newt MCN [h]	CHO SCE [i]	HeLa SCGE [j]	Skin Tumor Ind. [k]	SOS Tox [l]	Newt Tox [m]	CHO %C½ [n]	CHO SCGE [o]
CAN	1	1	1	1	1	1	1	1	1	1	1	1	1
DCAN	450	0.7	0.07	2.1	1.3	0.48	1.05	10	0.4	10	5	1.19	0.22
TCAN	450	0.2	0.01	36.7	0.98	0.42	3.61	10	0.9	100	50	0.43	0.60
BAN	–	–	–	–	1.03	2.42	–	10	–	33.3	20	21.3	15.61
DBAN	3000	0.5	6.2	3.4	1.78	1.15	32.5	100	1.4	33.3	10	24.0	12.81
BCAN	2300	0.9	2.2	6.3	1.92	0.46	13.0	–	1.1	66.7	40	8.1	1.86

[a] Data normalized to CAN = 1.0. Adapted from (Daniel et al. 1986; Lin et al. 1986). Data from (Le Curieux et al. 1995; Bull et al. 1985; Muller-Pillet et al. 2000; Pereira, Lin, and Mattox 1984) (Muellner et al. 2007), and this study.

[b] Abbreviations are HAN = haloacetonitriles, CAN = chloroacetonitrile, DCAN = dichloroacetonitrile, TCAN = trichloroacetonitrile, BAN = bromoacetonitrile, DBAN = dibromoacetonitrile, and BCAN = bromochloroacetonitrile.

[c] Measured as the *in vitro* inhibition of rat hepatic microsomal dimethylnitrosamine-demethylase (Lin et al. 1986).

[d] Extent metabolized measured as the percentage of the dose excreted as urinary thiocyanate (Pereira, Lin, and Mattox 1984).

[e] Alkylation potential measured as the ability to alkylate 4-(*p*-nitrobenzyl) pyridine (Daniel et al. 1986).

[f] DNA strand breakage potential in CCRF CEM cells determined by the alkaline unwinding method (Daniel et al. 1986).

[g] SOS chromotest (*Escherichia coli* PQ37) (Le Curieux et al. 1995).

[h] *Pleurodeles* walt1 larvae micronuclei induction (Le Curieux et al. 1995).

[i] CHO cell sister chromatid exchange induction (Bull et al. 1985).

[j] Single cell gel electrophoresis in HeLa S3 cells (Muller-Pillet et al. 2000).

[k] Measured as the total number of skin tumors per mouse after a topical dose of 2.4 g/kg of the HAN followed by 12-*O*-tetradacanoylphorbol-13-acetate for 20 weeks, data from (Bull et al. 1985); ranking from (Daniel et al. 1986).

[l] Threshold toxicity in the SOS chromotest (Le Curieux et al. 1995).

[m] Threshold toxicity in the newt micronucleus assay (Le Curieux et al. 1995).

[n] CHO cell chronic cytotoxicity %C½ values from this study normalized to CAN = 1.

[o] CHO cell SCGE genotoxic potency values from this study normalized to CAN = 1.

The potential of the haloacetonitriles to undergo nucleophilic addition at the partially positively charged carbon of the cyano group is dependent on the degree of halogenation. Polyhalogenation at the α carbon provides the ideal situation because (i) the halogens withdraw electrons away from the cyano carbon, making it more electrophilic, and (ii) the halogens have lesser tendency to leave. Data showed that trichloroacetonitrile is the most potent in this respect (Lin and Guion 1989). In an analogy to the halomethanes, the S_N2 reactivity would be expected to significantly contribute to the genotoxic potential of the haloacetonitriles. In the present study, the observed relative order of dibromoacetonitrile > iodoacetonitrile ≈ bromoacetonitrile > bromochloroacetonitrile > chloroacetonitrile > trichloroacetonitrile > dichloroacetonitrile for SCGE genotoxic potency is generally in agreement with the S_N2 SAR expectation. The higher activity of trichloroacetonitrile than dichloroacetonitrile may suggest that nucleophilic addition at the cyano carbon could also make some contribution to the genotoxicity. The toxic and genotoxic consequence of haloacetonitrile interaction with cellular macromolecules may be significantly affected by the presence of GSH and SH compounds. For mono- and trihaloacetonitriles, GSH conjugation is expected to be detoxifying because of elimination of reactive electrophiles. There is evidence that the toxicity and genotoxicity of these compounds may not be fully manifested until the cellular GSH pool is depleted (Abdelaziz et al. 1993). For dihaloacetonitriles, GSH conjugation is only detoxifying if both halogens are displaced. If only one is displaced, GSH conjugation can become an activation pathway because the resulting intermediate (an α-halothioether) is a highly reactive electrophile. There are many examples of dihaloalkanes being activated by GSH conjugation (Woo, Arcos, and Lai 1988). The relative importance of the GSH activation pathway for dihaloacetonitriles remains to be studied.

CHAPTER 7
HALOACETAMIDES

INTRODUCTION

Haloacetamides have been identified as DBPs from drinking water treatment plants and from laboratory studies (Stevens et al. 1990; Richardson et al. 1999, 1999). Thirteen haloacetamides were evaluated in this study, bromoacetamide, bromochloroacetamide, bromodichloroacetamide, bromoiodoacetamide, chloroacetamide, chloroiodoacetamide, dibromoacetamide, dibromochloroacetamide, dichloroacetamide, diiodoacetamide, iodoacetamide, tribromoacetamide, and trichloroacetamide (Table 2.1). Also in this study a new iodinated acetamide, bromoiodoacetamide was discovered as a DBP and its occurrence was measured.

OCCURRENCE

Haloacetamides were recently quantified in a Nationwide Occurrence Study of priority, unregulated DBPs (Krasner et al. 2006). Chloro-, bromo-, dichloro-, dibromo-, and trichloroacetamide were found in finished drinking water from several locations (maximum of 14 µg/L) from water treated with chlorine dioxide-chlorine-chloramines. As with the formation of haloacetonitriles (Muellner et al. 2007), there is preliminary evidence that chloramination may increase their formation. Because nitriles can hydrolyze to form haloacetamides (Exner, Burk, and Kyriacou 1973; Bruice 1995), it is possible that the haloacetamides are hydrolysis products of the corresponding haloacetonitriles, which are commonly found as DBPs (Richardson et al. 2007).

Drinking Water Analysis

Drinking water samples were shipped cold, overnight to Dr. Susan Richardson at the U.S. EPA for analysis. Samples were extracted on the day they were received. One liter of drinking water (finished water) and raw, untreated water were extracted by a method similar to EPA Method 552.3. Water samples were acidified to pH 0.5 with sulfuric acid, 400 g of sodium sulfate was added to water samples (for salting out of analytes), and water samples were extracted two times with 200 mL of methyl *tert*-butyl ether (MTBE) using a separatory funnel. Excess water was removed with the separatory funnel and with sodium sulfate, and the MTBE extract was vacuum-evaporated to 1 mL. Drinking water samples were collected from full-scale drinking water treatment plants in the United States that use chloramines for disinfection. One plant used chlorine for disinfection.

GC/MS Analysis

Gas chromatography/mass spectrometry (GC/MS) analyses with electron ionization (EI) were performed on a Waters-Micromass Autospec II high resolution, double focusing mass spectrometer at 1000 resolution equipped with an Agilent 6890 gas chromatograph. The mass spectrometer was operated at an accelerating voltage of 8 kV. Perfluorokerosene was used as the mass calibrant. Injections of 1 µL of the extracts (or standard solutions in MTBE) were introduced via a splitless injector onto a J&W-Agilent Scientific DB-5 column (30 m, 0.25 mm i.d.,

0.25 μm film thickness). The GC temperature program consisted of an initial temperature of 35°C for 4 min, followed by a rate increase of 9°C /min to 285°C, which was held for 30 min. A GC injection port temperature of 250°C and a GC/MS transfer line temperature of 285°C were used.

Identification and Occurrence of a New Iodinated Acetamide

Bromoiodoacetamide was identified as a DBP for the first time in drinking water from 12 of 23 treatment plants analyzed that were located in 6 U.S. states (Plewa, Muellner et al. 2008). One plant used chlorine for disinfection; 22 plants used chloramination. These plants had source waters with relatively high natural bromide and iodide levels. Where bromoiodoacetamide was identified, three plants had very small amounts in their raw waters, at levels 500× lower than the finished waters. The other 9 plants exhibited bromoiodoacetamide only in their finished waters. Iodoacetamide, chloroiodoacetamide, or diiodoacetamide were not detected using GC with selected ion monitoring-MS.

The EI mass spectrum of bromoiodoacetamide is shown in Figure 7.1. Selected ion monitoring of 5 key ions (m/z 127, 136, 138, 220, and 263) were used to identify this compound in the drinking water extracts. A match of these ions with a match of the GC retention time was used to confirm its presence. All haloacetamides measured expressed distinctive GC/MS chromatographic peak shapes (Plewa, Muellner et al. 2008). This distinctive 'tailing' peak shape appears to be due to surface reactions of the haloacetamides in the EI ion source and provided further confirmation of bromoiodoacetamide (Figure 7.2). All of the haloacetamides show a prominent peak at m/z 44, which represents the amide group (Figure 7.1). The presence of bromine and iodine is evident in the mass spectrum of bromoiodoacetamide, with 1-bromine doublets present at m/z 263/265, 220/222, and 136/138, and the presence of iodine at m/z 127 and loss of iodine at m/z 136/138.

Chlorinated and brominated forms of this acetamide have been measured in drinking water previously (Krasner et al. 2006), but this research, for the first time discovered an iodinated amide DBP. Naturally occurring bromide and iodide contribute to the formation of brominated and iodinated DBPs (Richardson et al. 1999; Cemeli et al. 2006; Plewa, Wagner, Richardson et al. 2004; Thomas, Weisner, and Brass 1980; Krasner et al. 1989; Cancho et al. 2000; Richardson 2003). There is evidence that chloramination increases the formation of iodinated DBPs (Plewa, Wagner, Richardson et al. 2004; Bichsel and von Gunten 2000, 1999). Therefore, while this is the first report of an iodinated amide DBP, it is not surprising that iodo-amides would form in source waters with high bromide/iodide and chloramine disinfection. As mentioned earlier, it is possible that bromoiodoacetamide and other haloacetamides are hydrolysis products of the corresponding haloacetonitriles, which are commonly found as DBPs. The amides can undergo further hydrolysis to form carboxylic acids, but this reaction requires longer reaction times and higher temperatures than the initial conversion to the amide.

PREPARATION OF HALOACETAMIDES

Eight haloacetamides were synthesized specifically for this study by Dr. A. B. McKague at CanSyn Co. Dibromoacetamide was prepared from ethyl dibromoacetate by reaction with ammonium hydroxide (Taylor and Forscey 1930). Bromochloroacetamide was prepared from bromochloroacetic acid by conversion to the ethyl ester followed by reaction with ammonium

hydroxide (Taylor and Forscey 1930). Bromodichloroacetamide and dibromochloroacetamide were prepared from the corresponding acids (Neumeister 1882; Zimmer, Amer, and Rahi 1990) by conversion to the methyl esters with BF_3/methanol followed by reaction with ammonium hydroxide (Neumeister 1882). Tribromoacetamide was prepared from the acid in a similar manner. Bromoiodoacetamide was similarly prepared from bromoiodoacetic acid to give colorless material, mp 181-183°C. The purity of the product by gas chromatography using flame ionization detection was 85% and contained 7.5% each of dibromoacetamide and diiodoacetamide as impurities. Chloroiodoacetamide was prepared from methyl chloroiodoacetate (Seyferth and Woodruff 1974) and diiodoacetamide was prepared from diiodoacetic acid (Cobb 1958) via the methyl ester, by similar reaction with ammonium hydroxide.

GENOTOXICITY

There is little information on the genotoxicity and carcinogenicity of haloamides. These agents react with cellular protein thiols and are prototypical alkylating agents inducing a multitude of biological responses, including apoptosis and necrosis (van De Water et al. 1999). Iodoacetamide was a cocarcinogen in a mouse skin assay (Gwynn and Salaman 1953) and enhanced nitrosamide-induced tumors in rats (Takahashi et al. 1976; Fukushima et al. 1977).

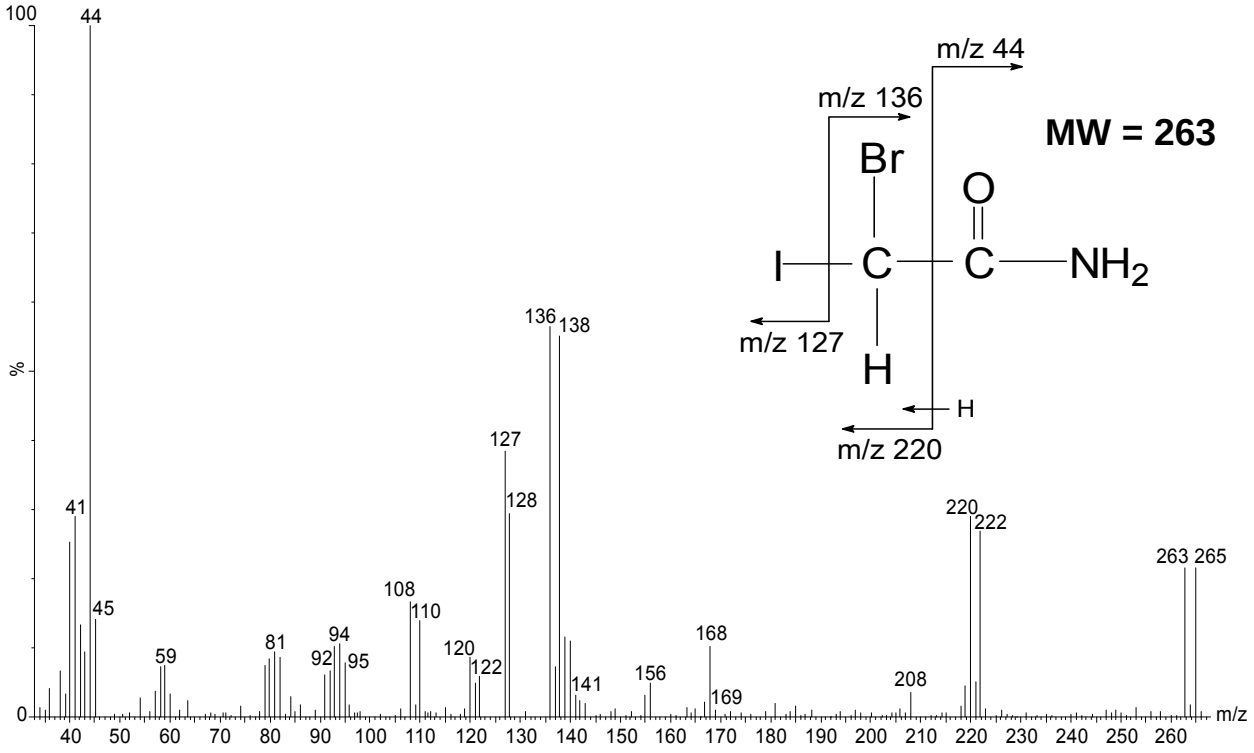


Figure 7.1 EI mass spectrum of bromoiodoacetamide

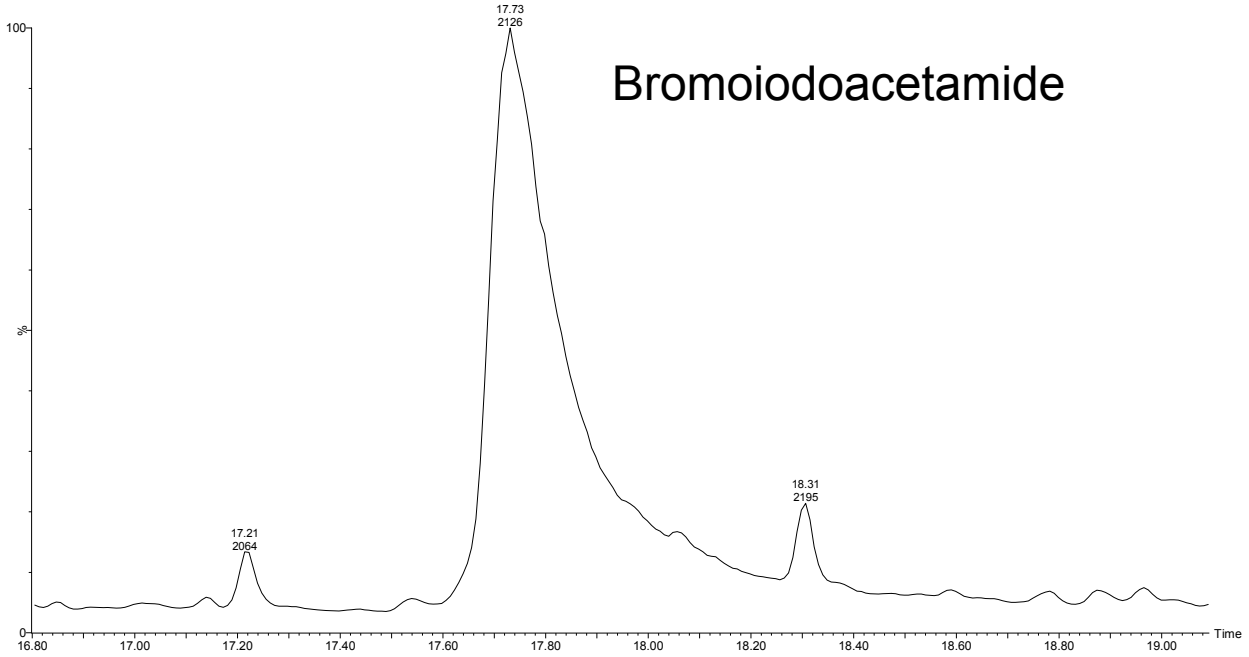


Figure 7.2 GC/MS chromatogram of a coinjection of bromoiodoacetamide and a drinking water extract

CHO CELL CYTOTOXICITY ANALYSIS OF THE HALOACETAMIDES

The CHO cell chronic cytotoxicity of the 13 haloacetamides analyzed in this study are presented in Table 7.1. 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 CHO cell chronic cytotoxicity concentration-response curves are presented for each haloacetamide in the following figures: bromoacetamide (Figure 7.3), bromochloroacetamide (Figure 7.4), bromodichloroacetamide (Figure 7.5), bromoiodoacetamide (Figure 7.6), chloroacetamide (Figure 7.7), chloroiodoacetamide (Figure 7.8), dibromoacetamide (Figure 7.9), dibromochloroacetamide (Figure 7.10), dichloroacetamide (Figure 7.11), diiodoacetamide (Figure 7.12), iodoacetamide (Figure 7.13), tribromoacetamide (Figure 7.14), and trichloroacetamide (Figure 7.15). A comparison of the relative cytotoxicity of the haloacetamides analyzed in this study is presented in Figure 7.16.

The lowest concentration that induced a significant cytotoxic response ranged from 25 nM (diiodoacetamide) to 800 µM (dichloroacetamide) (Table 7.1). 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 > dibromochlo-

roacetamide > chloroiodoacetamide > bromodichloroacetamide > dibromoacetamide > bromochloroacetamide > chloroacetamide > dichloroacetamide > trichloroacetamide.

Table 7.1
CHO cell chronic cytotoxicity of the haloacetamide DBPs and related chemicals

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.

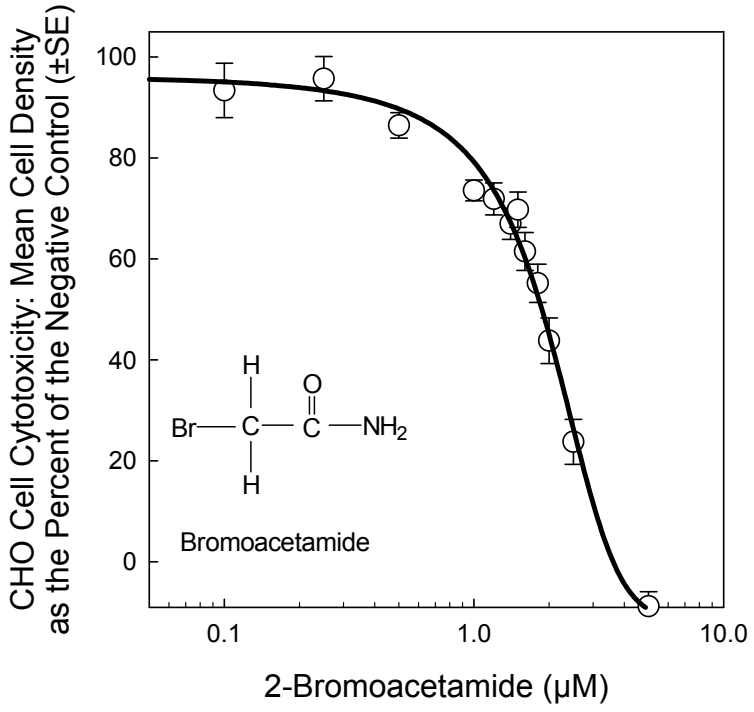


Figure 7.3 CHO cell chronic cytotoxicity concentration-response curve for bromoacetamide

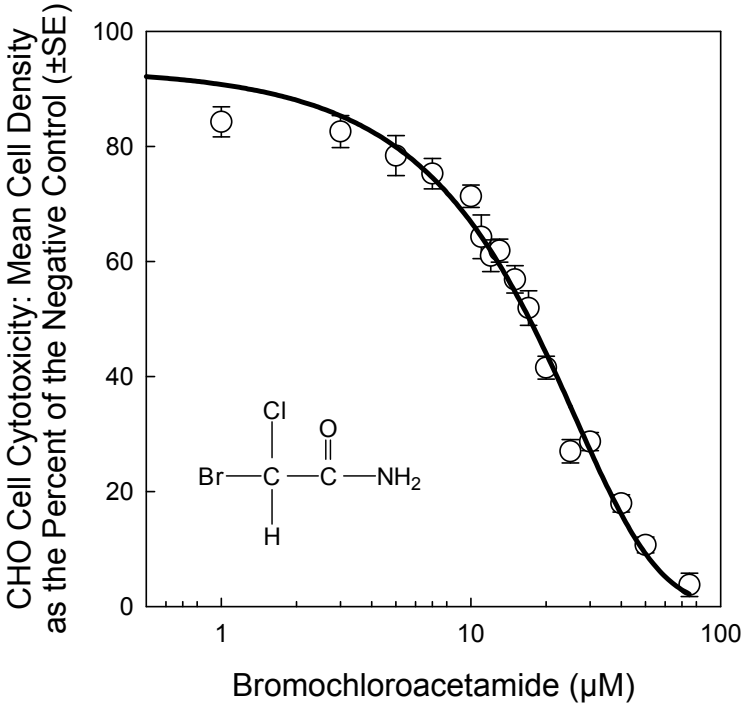


Figure 7.4 CHO cell chronic cytotoxicity concentration-response curve for bromochloroacetamide

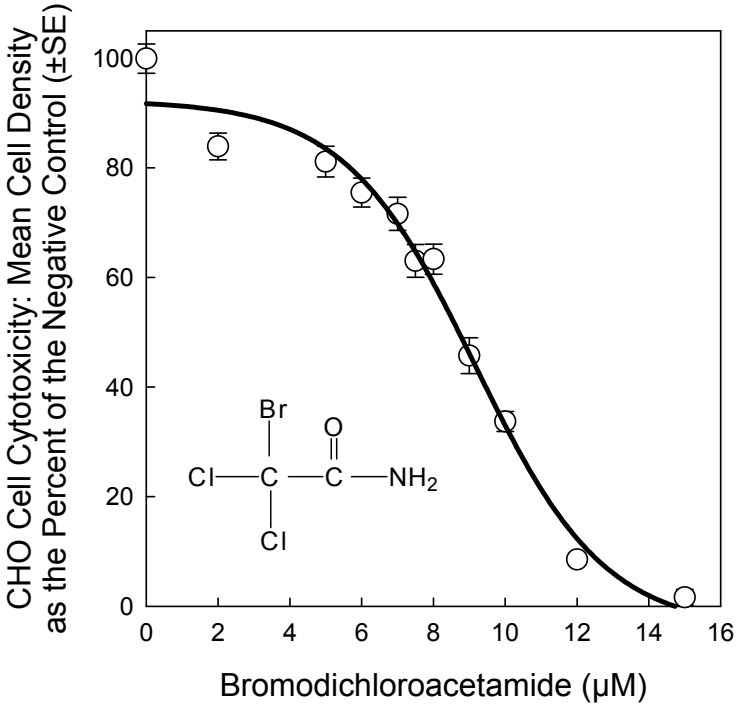


Figure 7.5 CHO cell chronic cytotoxicity concentration-response curve for bromodichloroacetamide

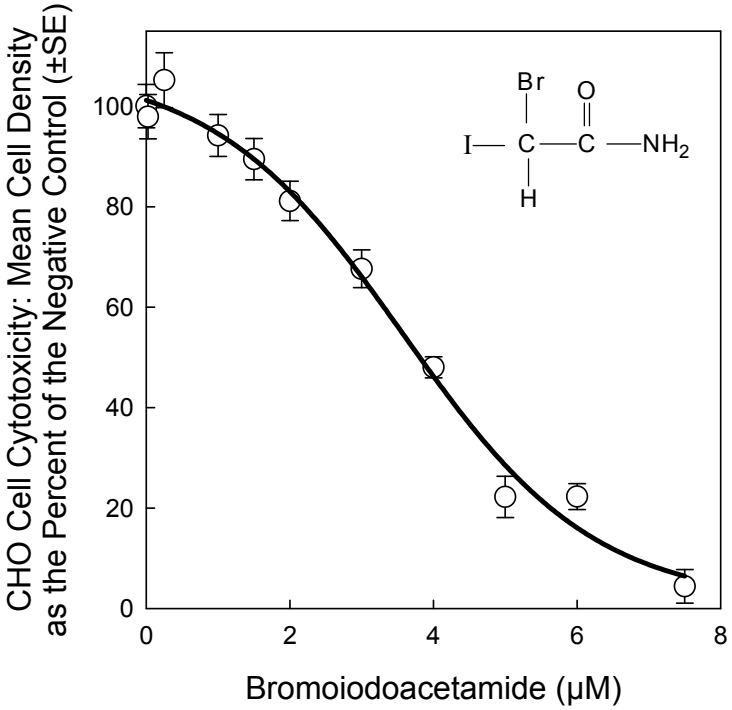


Figure 7.6 CHO cell chronic cytotoxicity concentration-response curve for bromoiodoacetamide

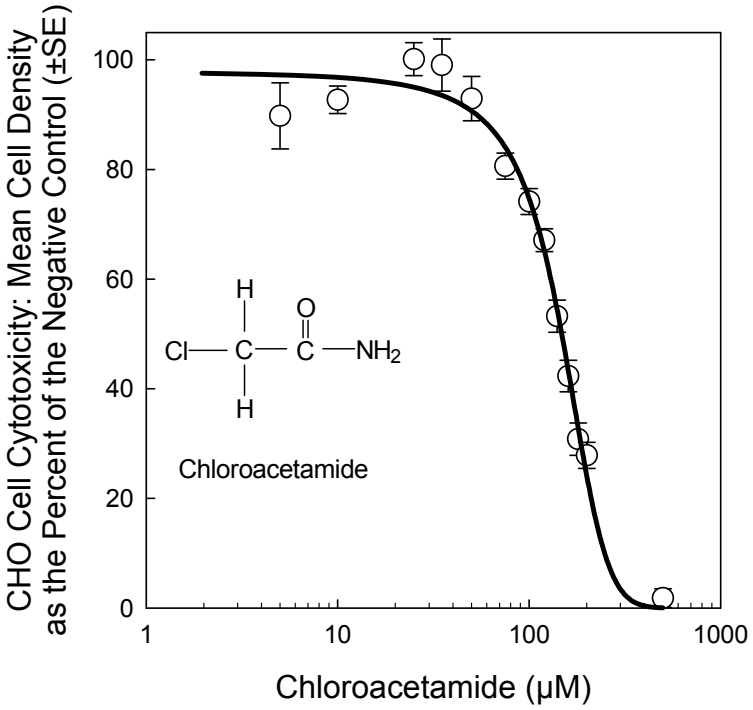


Figure 7.7 CHO cell chronic cytotoxicity concentration-response curve for chloroacetamide

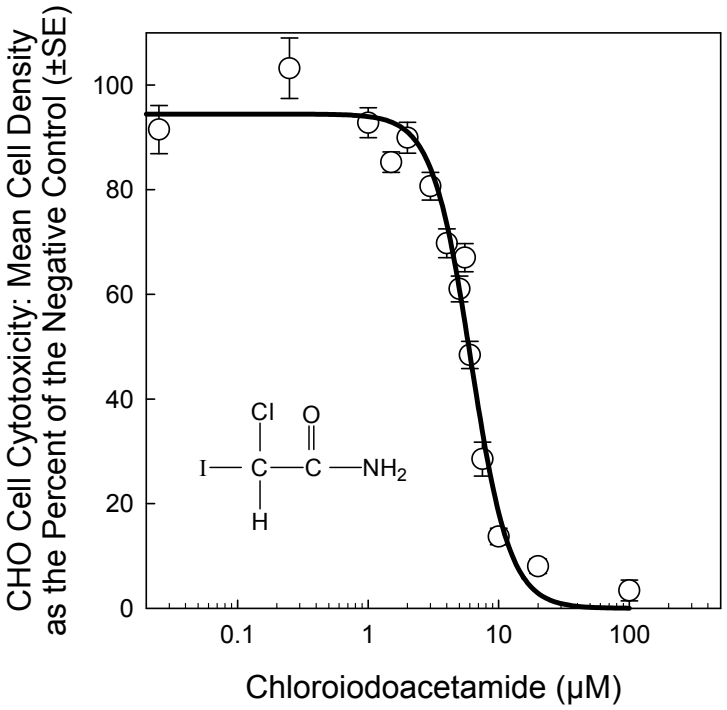


Figure 7.8 CHO cell chronic cytotoxicity concentration-response curve for chloroiodoacetamide

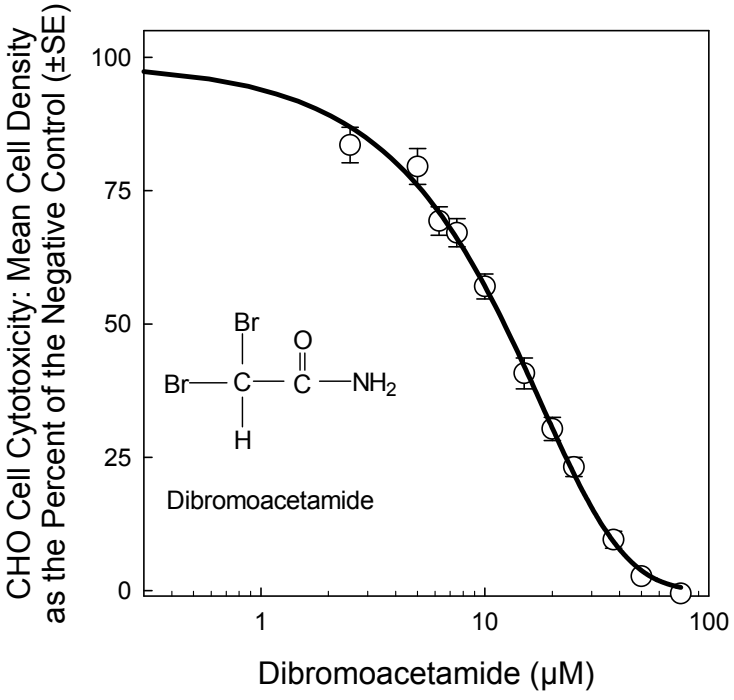


Figure 7.9 CHO cell chronic cytotoxicity concentration-response curve for dibromoacetamide

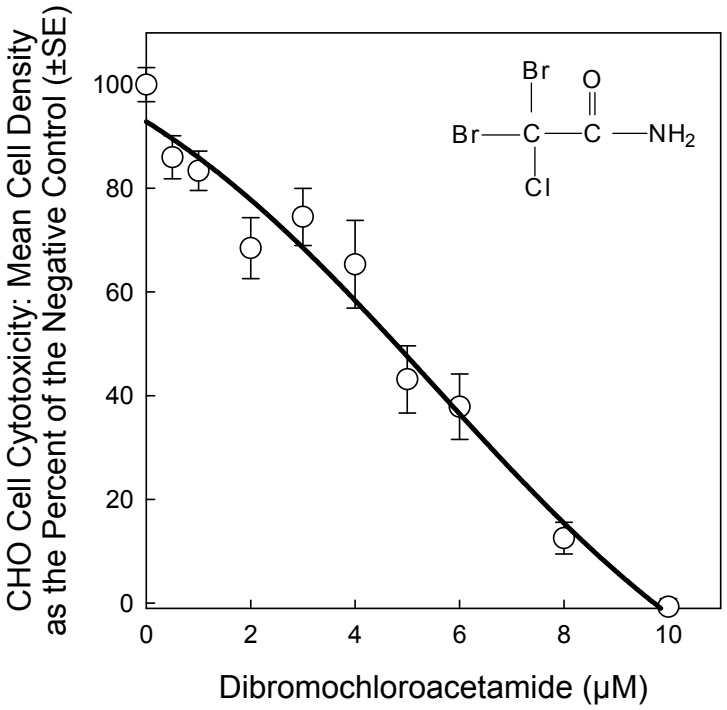


Figure 7.10 CHO cell chronic cytotoxicity concentration-response curve for dibromochloroacetamide

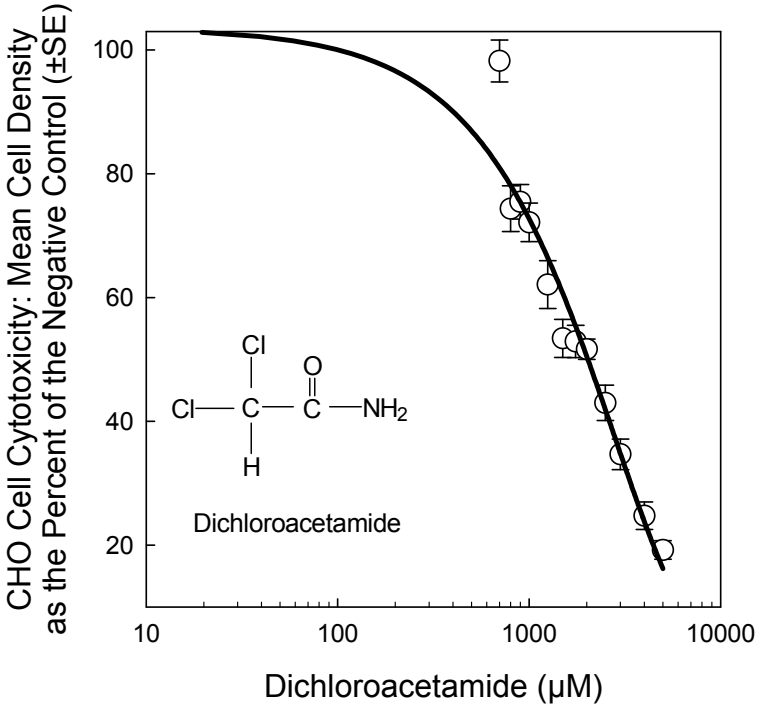


Figure 7.11 CHO cell chronic cytotoxicity concentration-response curve for dichloroacetamide

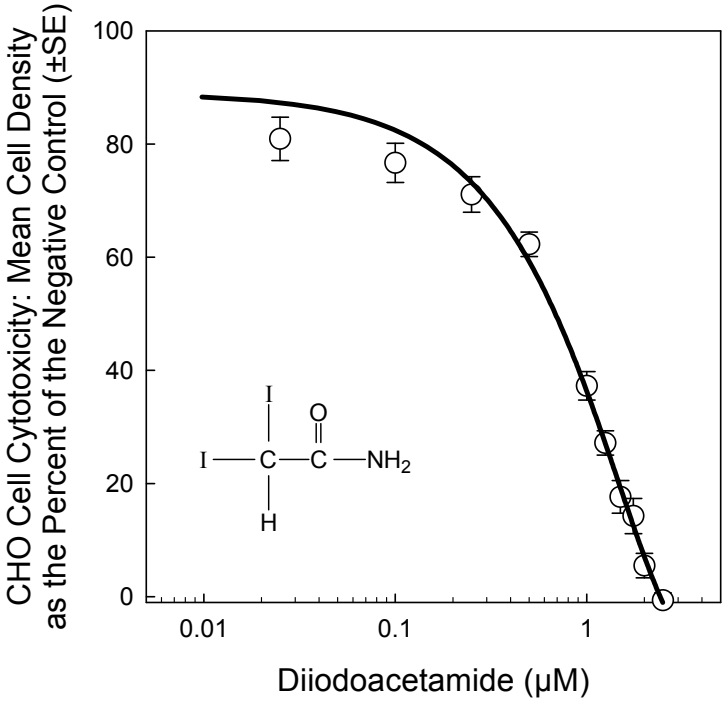


Figure 7.12 CHO cell chronic cytotoxicity concentration-response curve for diiodoacetamide

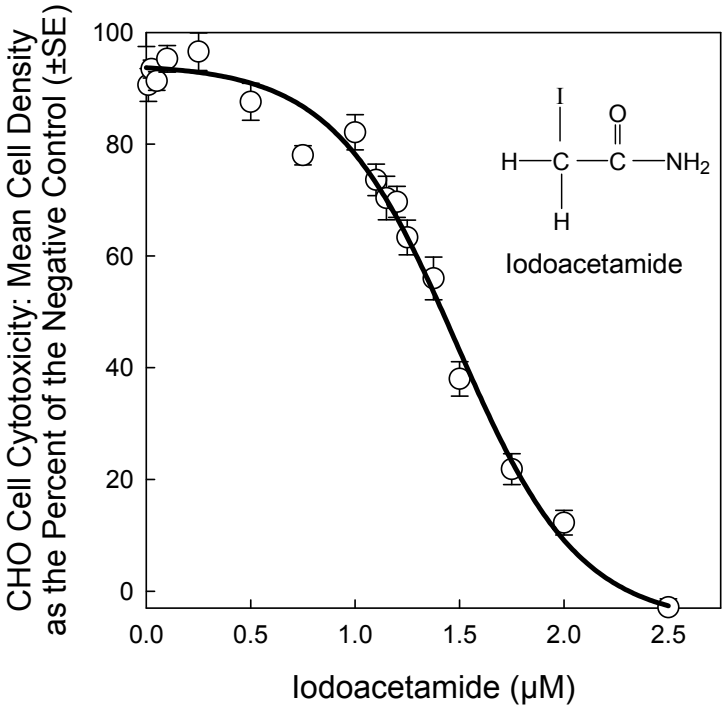


Figure 7.13 CHO cell chronic cytotoxicity concentration-response curve for iodoacetamide

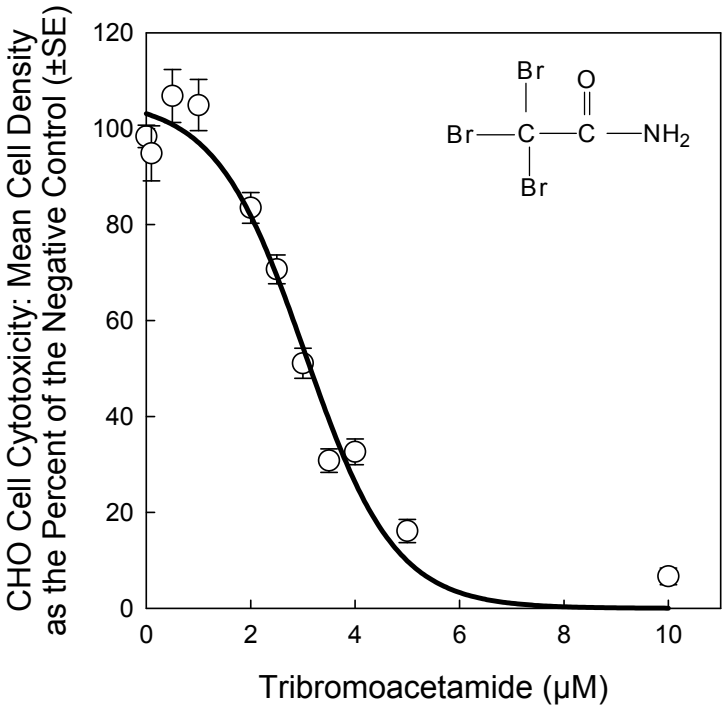


Figure 7.14 CHO cell chronic cytotoxicity concentration-response curve for tribromoacetamide

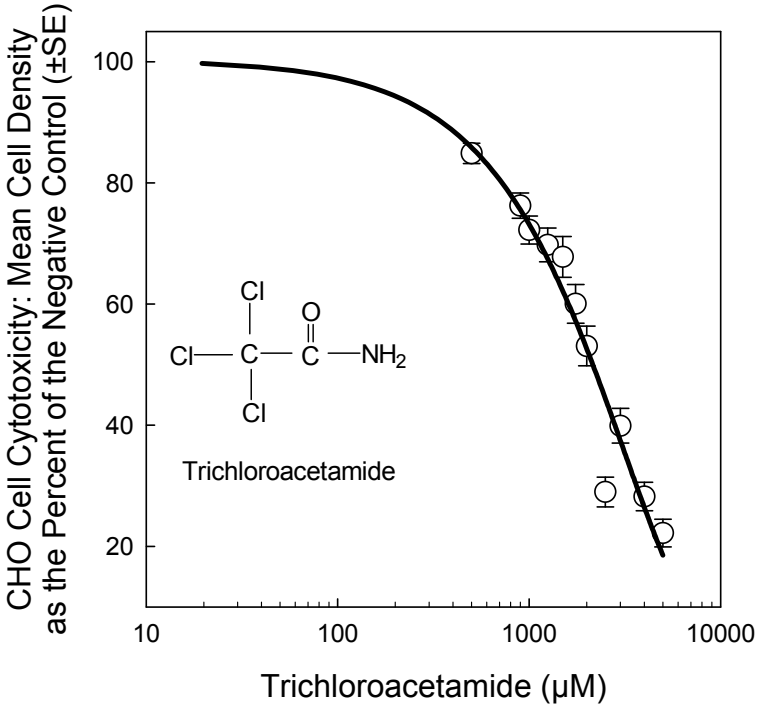


Figure 7.15 CHO cell chronic cytotoxicity concentration-response curve for trichloroacetamide

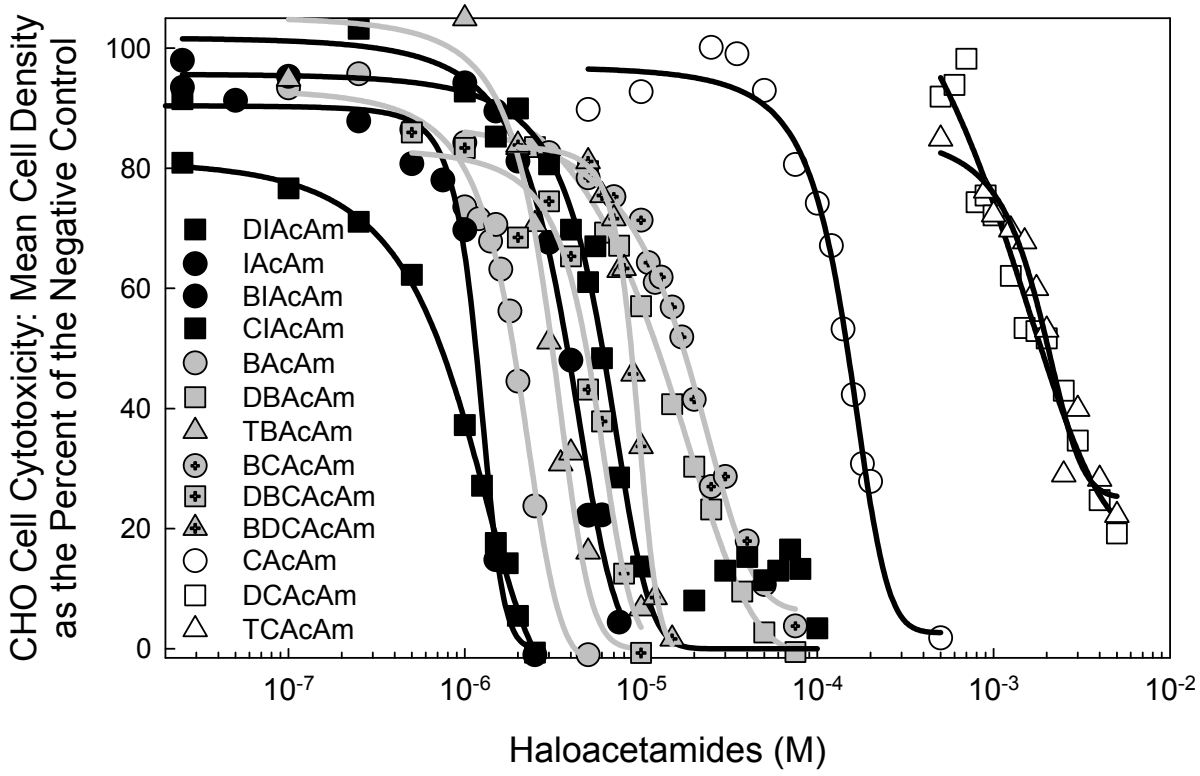


Figure 7.16 Comparison of the CHO cell chronic cytotoxicity concentration-response curves for the haloacetamides analyzed in this study. The abbreviations are DIAcAm = diiodoacetamide, IAcAm = iodoacetamide, BIAcAm = bromoiodoacetamide, CIAcAm = chloroiodoacetamide, BAcAm = bromoacetamide, DBAcAm = dibromoacetamide, TBAcAm = tribromoacetamide, BCAcAm = bromochloroacetamide, DBCAcAm = dibromochloroacetamide, BDCAcAm = bromodichloroacetamide, CAcAm = chloroacetamide, DCAcAm = dichloroacetamide, and TCAcAm = trichloroacetamide

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 7.2. 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.

Table 7.2
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.

The CHO cell concentration-response curves illustrating the induction of genomic DNA damage are presented for bromoacetamide, bromochloroacetamide, bromodichloroacetamide, bromoiodoacetamide, chloroacetamide, chloroiodoacetamide, dibromoacetamide, dibromochloroacetamide, dichloroacetamide, diiodoacetamide, iodoacetamide, tribromoacetamide, and trichloroacetamide in Figures 7.17 to 7.29, respectively. A comparison of the CHO cell genotoxicity for these agents is presented in Figure 7.30.

The lowest concentration that induced a significant SCGE genotoxic response ranged from 25 μM for diiodoacetamide, bromoiodoacetamide, bromoacetamide, or dibromochloroacetamide to 5 mM for trichloroacetamide. The SCGE genotoxic potency value ranged from 32.5 μM for tribromoacetamide to 6.5 mM for trichloroacetamide (Table 7.2). The rank order of genotoxic potency from most potent to least was tribromoacetamide > diiodoacetamide ≈ iodoacetamide > bromoacetamide > dibromochloroacetamide > bromoiodoacetamide > bromodichloroacetamide > chloroiodoacetamide > bromochloroacetamide > dibromoacetamide > chloroacetamide > trichloroacetamide. Dichloroacetamide was not genotoxic (Table 7.2).

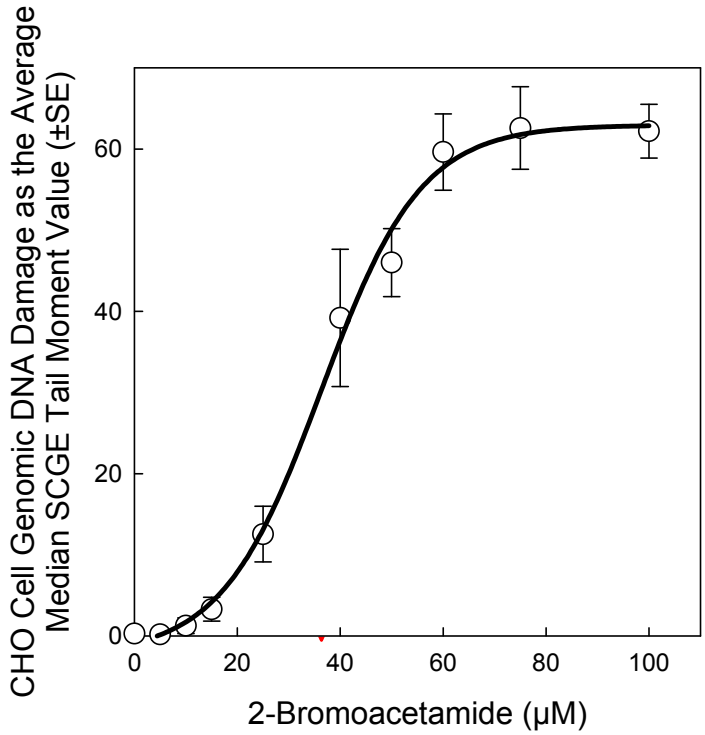


Figure 7.17 CHO cell SCGE genotoxicity concentration-response curve for bromoacetamide

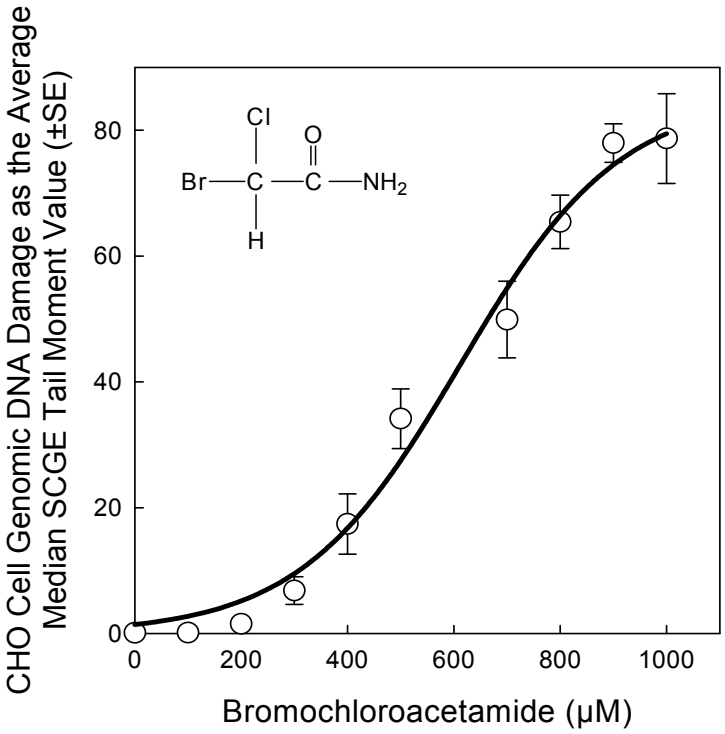


Figure 7.18 CHO cell SCGE genotoxicity concentration-response curve for bromochloroacetamide

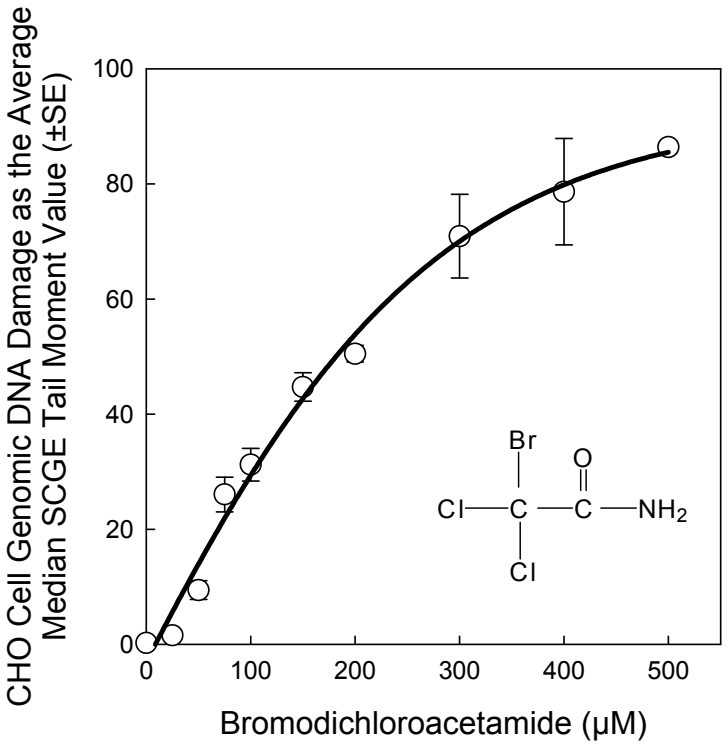


Figure 7.19 CHO cell SCGE genotoxicity concentration-response curve for bromodichloroacetamide

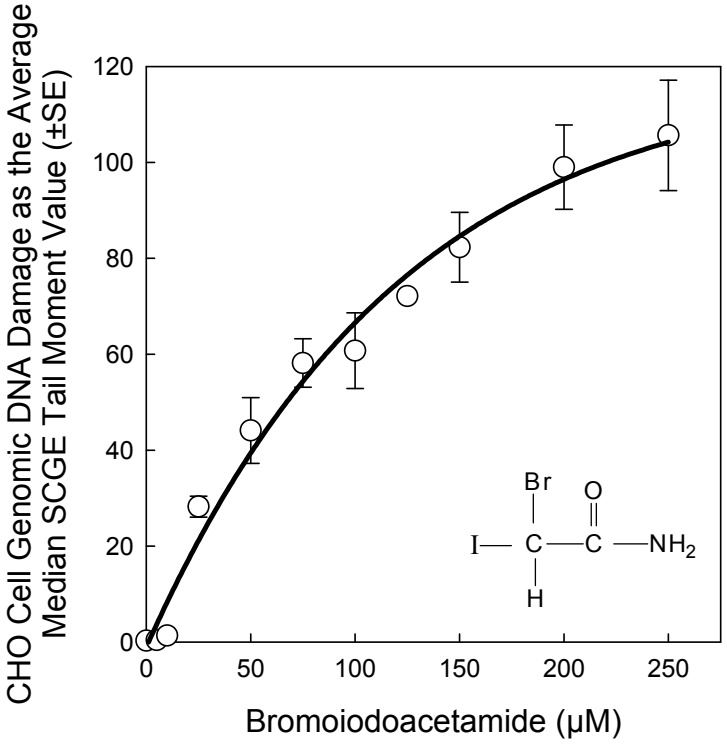


Figure 7.20 CHO cell SCGE genotoxicity concentration-response curve for bromoiodoacetamide

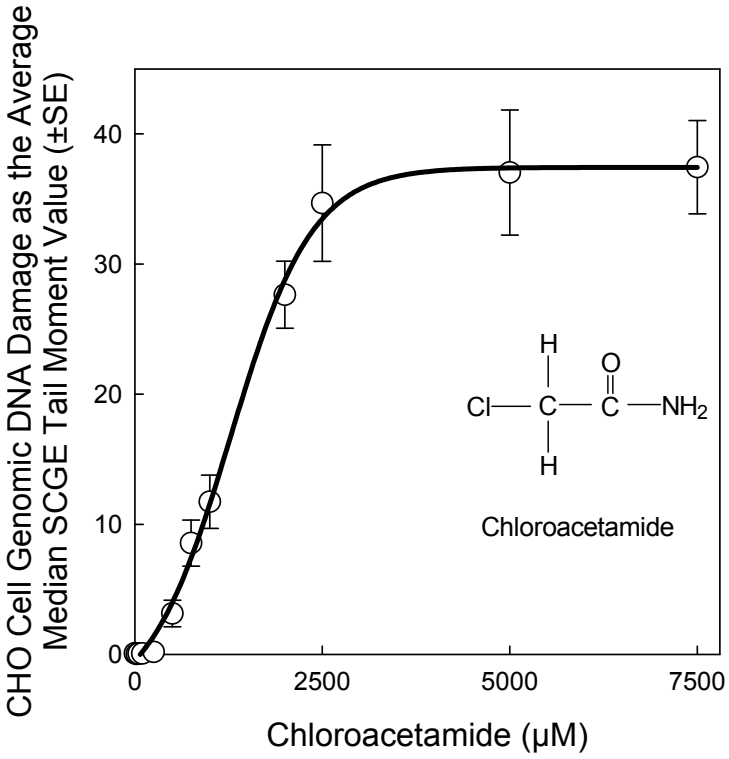


Figure 7.21 CHO cell SCGE genotoxicity concentration-response curve for chloroacetamide

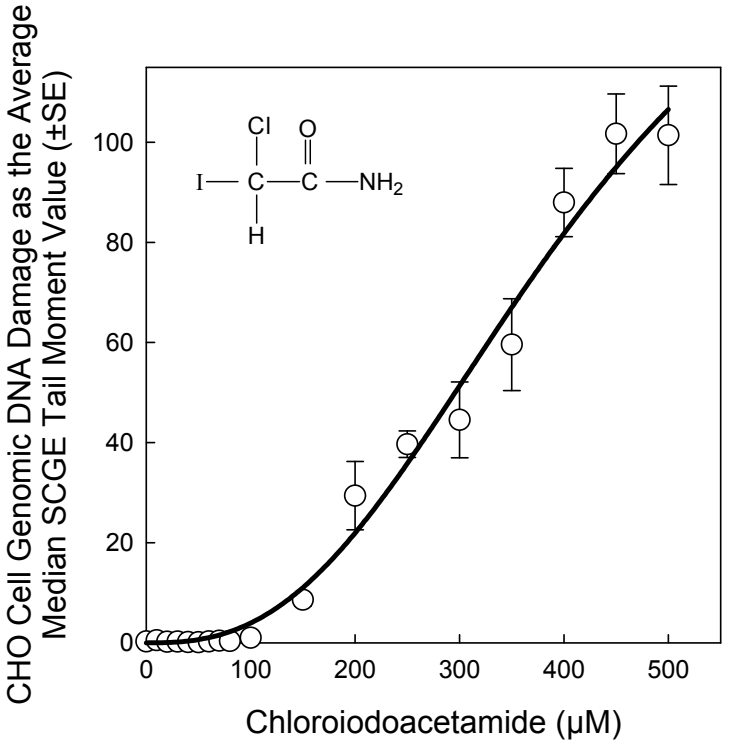


Figure 7.22 CHO cell SCGE genotoxicity concentration-response curve for chloroiodoacetamide

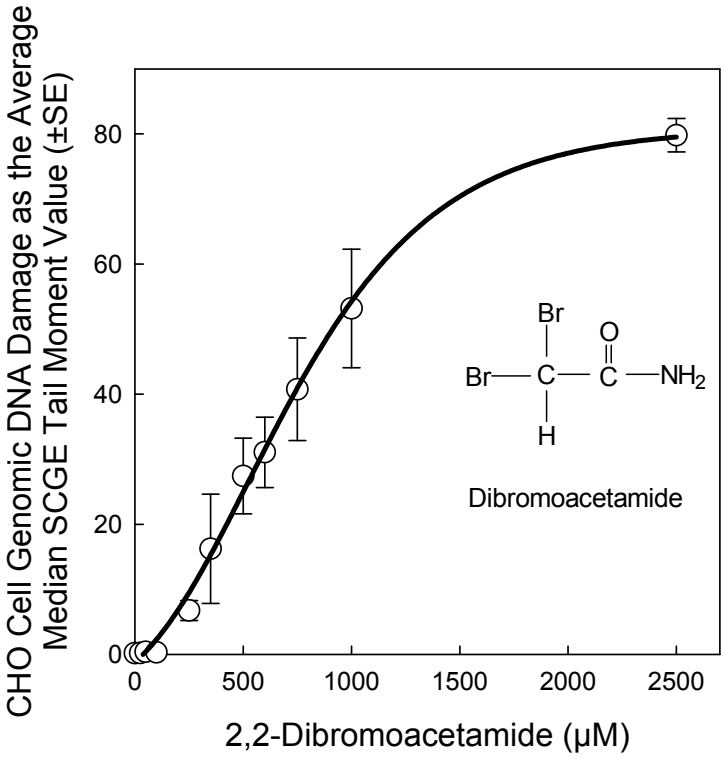


Figure 7.23 CHO cell SCGE genotoxicity concentration-response curve for dibromoacetamide

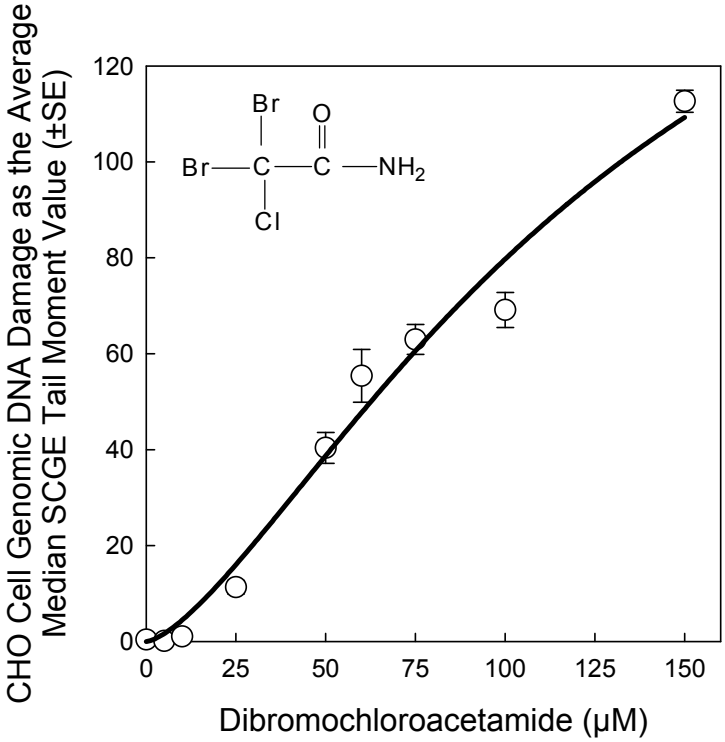


Figure 7.24 CHO cell SCGE genotoxicity concentration-response curve for dibromochloroacetamide

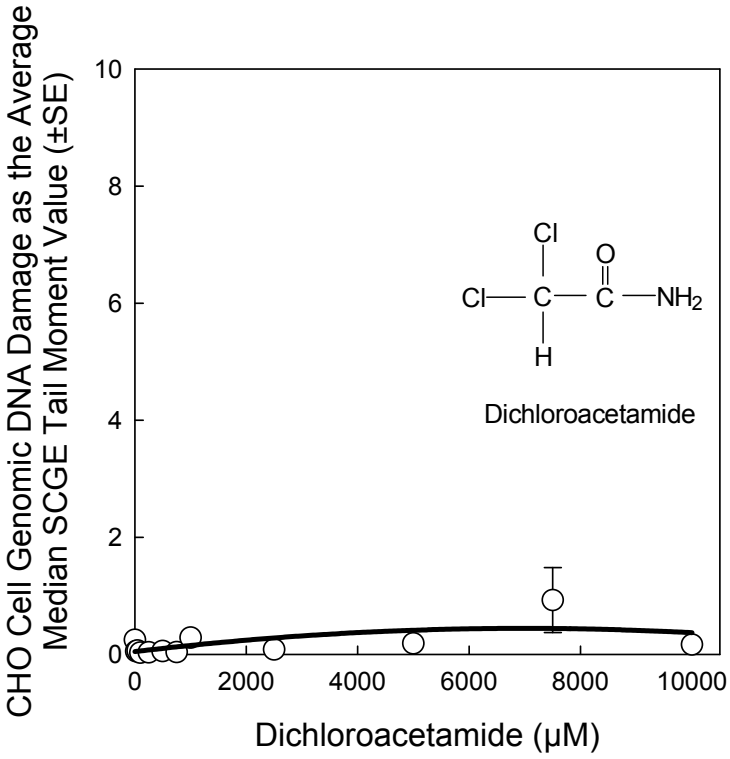


Figure 7.25 CHO cell SCGE genotoxicity concentration-response curve for dichloroacetamide

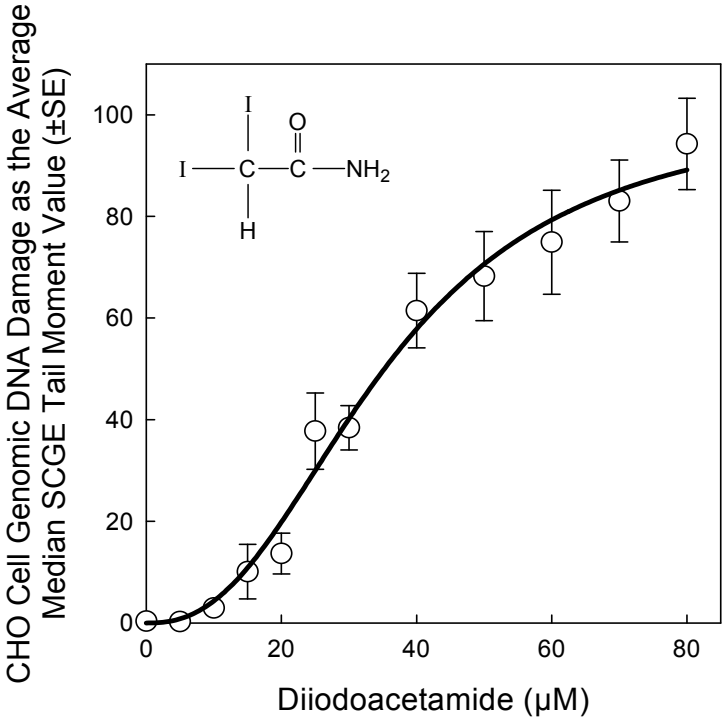


Figure 7.26 CHO cell SCGE genotoxicity concentration-response curve for diiodoacetamide

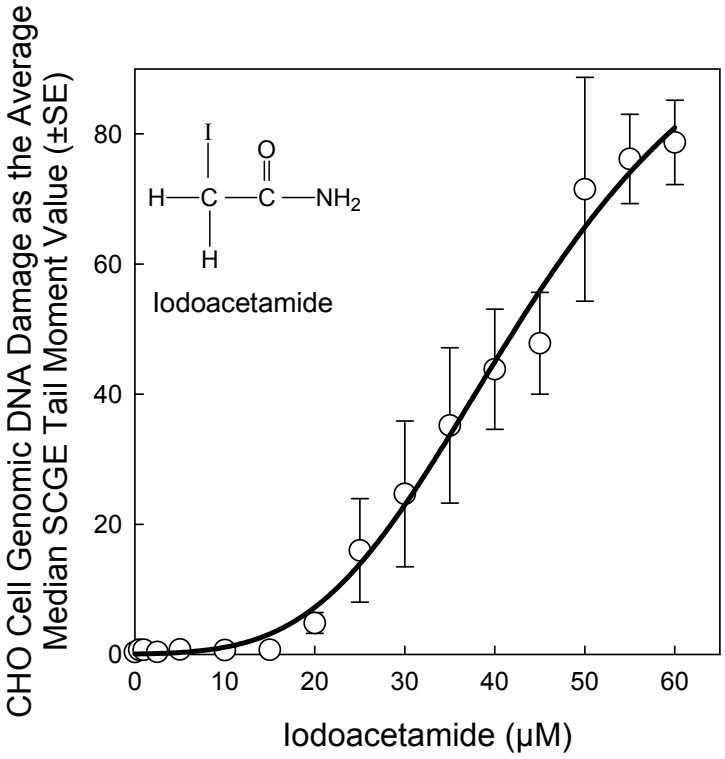


Figure 7.27 CHO cell SCGE genotoxicity concentration-response curve for iodoacetamide

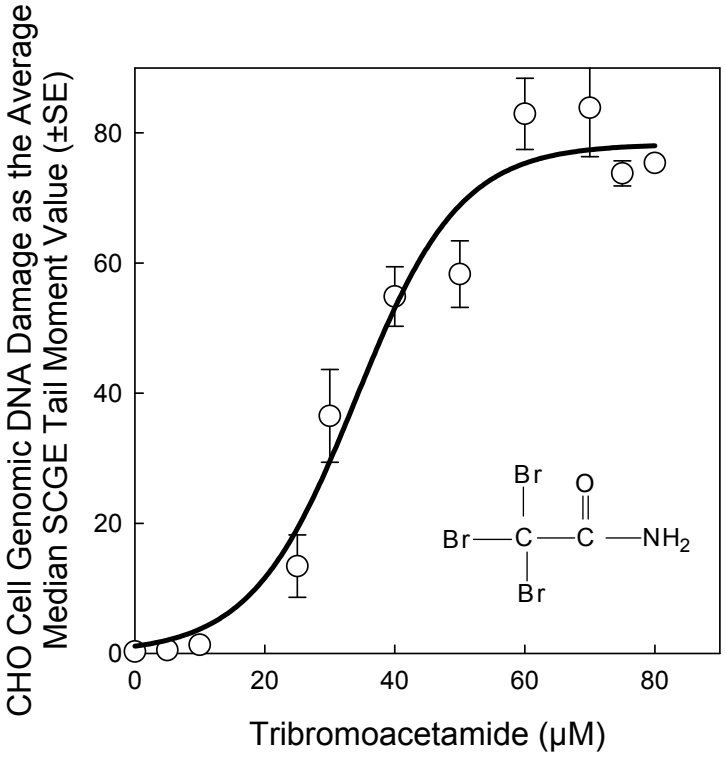


Figure 7.28 CHO cell SCGE genotoxicity concentration-response curve for tribromoacetamide

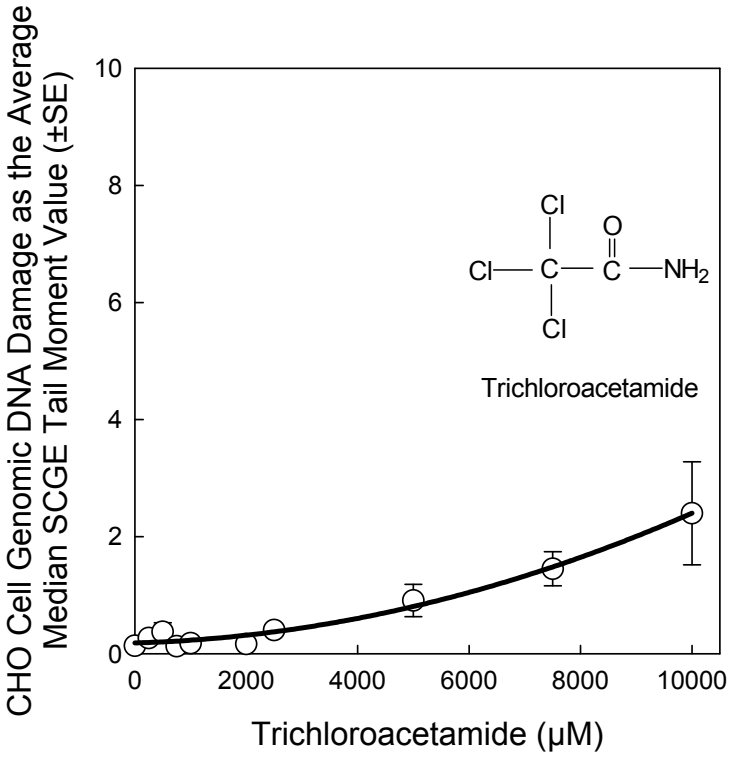


Figure 7.29 CHO cell SCGE genotoxicity concentration-response curve for trichloroacetamide

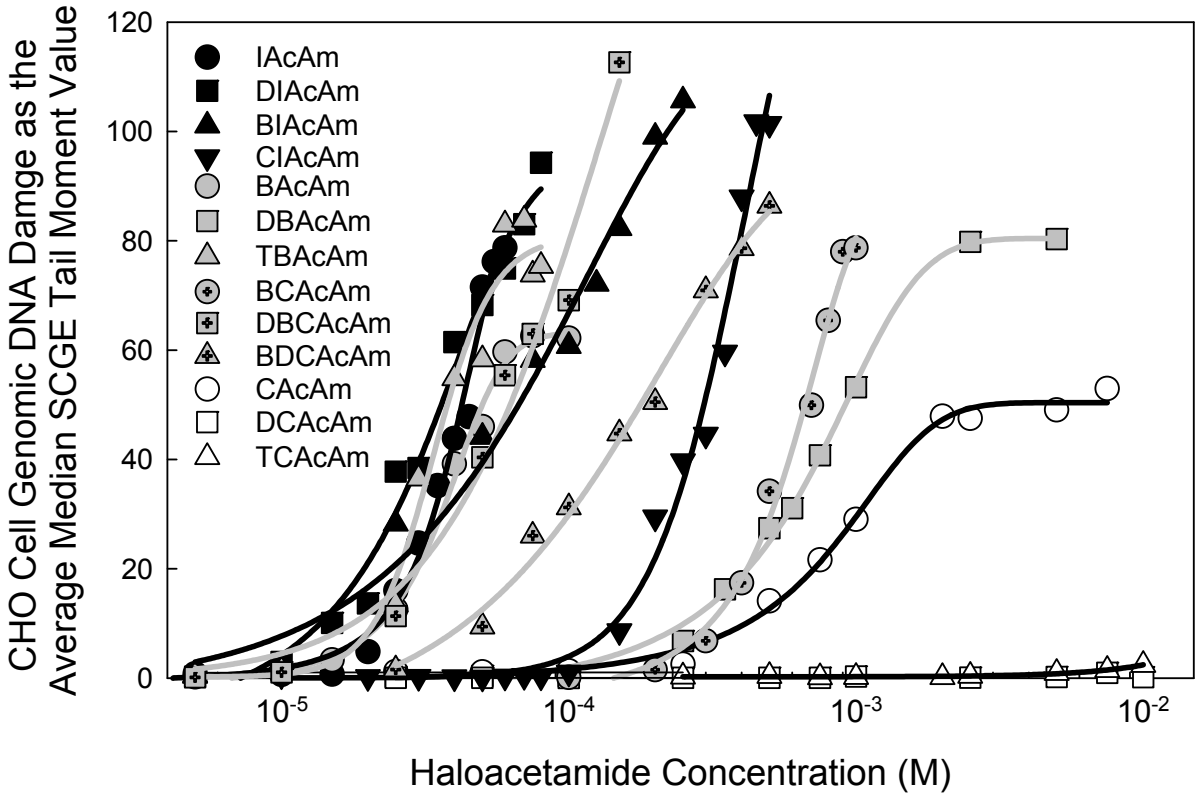


Figure 7.30 Comparison of the SCGE genotoxicity concentration-response curves of 13 haloacetamides. The abbreviations are IAcAm = iodoacetamide, DIAcAm = diiodoacetamide, BIAcAm = bromoiodoacetamide, CIAcAm = chloroiodoacetamide, BAcAm = bromoacetamide, DBAcAm = dibromoacetamide, TBAcAm = tribromoacetamide, BCAcAm = bromochloroacetamide, DBCAcAm = dibromochloroacetamide, BDCAcAm = bromodichloroacetamide, CAcAm = chloroacetamide, DCAcAm = dichloroacetamide, and TCAcAm = trichloroacetamide

COMPARATIVE CYTOTOXICITY AND GENOTOXICITY OF THE HALOACETAMIDES

Structure-Activity Relationships (SAR) and Factors Affecting the Toxicity of Haloacetamides

The haloacetamides have or may generate a number of electrophilic reactivities: (i) for monohaloacetamides, alkylation by the S_N2 reaction, inducing the displacement of a halogen atom at the α carbon, (ii) for dihaloacetamides, the potential generation of highly reactive α-halothioether electrophilic intermediates by cellular glutathione GSH or –SH compounds, (iii) for trihaloacetamides, nucleophilic attack at the electrophilic carbonyl carbon to yield trihalomethyl carbanions, that may lead to trihalomethanes as well as electrophilic dihalocarbene intermediates. In addition to chemical reactivity, the capacity to cross cell membranes is an important factor for toxicity. The logarithm of the octanol-water partition coefficient (log P) is a measure of

lipophilicity which correlates with cell permeability. Estimated log P values are presented in Table 7.3. Log P increased with the degree of halogenation and with the size of the halogen.

For the 13 haloacetamides analyzed, CHO cell chronic cytotoxicity and genotoxicity were highly and significantly correlated ($r = 0.99$; $P < 0.001$). The rank order and relative activities for the monohaloacetamides (cytotoxicity and genotoxicity) are iodoacetamide > bromoacetamide >> chloroacetamide. For the cytotoxicity of the dihaloacetamides the rank order was diiodoacetamide > bromoiodoacetamide > chloroiodoacetamide > dibromoacetamide > bromochloroacetamide > > dichloroacetamide. The genotoxicity of the dihaloacetamides followed the order of diiodoacetamide > bromoiodoacetamide > chloroiodoacetamide > bromochloroacetamide > dibromoacetamide; dichloroacetamide was inactive. The rank order of the trihaloacetamides (cytotoxicity and genotoxicity) was tribromoacetamide > dibromochloroacetamide > bromodichloroacetamide >> trichloroacetamide.

The rank order and relative activity of the monohaloacetamides are related to their S_N2 reactivity. Owing to increasing bond length and decreasing dissociation energy, the leaving tendency of the halogen in alkyl halides followed the order, I > Br >> Cl. The S_N2 reactivity of an alkyl iodide was 3-5× greater than alkyl bromide, which was 50× greater than alkyl chloride (Plewa, Wagner, Richardson et al. 2004; Loudon 1995). The cytotoxicity of iodoacetamide was 1.3× greater than bromoacetamide, which was 78× greater than chloroacetamide. Iodoacetamide was more genotoxic than bromoacetamide, which was 38× more potent than chloroacetamide. It appears that log P does not play a major role; the small difference in the log P of bromoacetamide versus chloroacetamide cannot account for the large difference in relative activity (Table 7.3).

An approach to combining both the cytotoxicity and genotoxicity of an individual compound into a single metric was the development of the combined Toxicity Index value. The combined Toxicity Index value is the reciprocal of the averaged %C½ and the SCGE genotoxic potency values. A larger Toxicity Index value indicates greater overall toxicity. The combined Toxicity Indices of the haloacetamides are presented in Table 7.3. Consistent with the relative leaving tendencies of the halogen, dihaloacetamides containing one or two iodo group(s) expressed the greatest combined Toxicity Indices, followed by bromo group(s) and chloro group(s) (Table 7.3). Dichloroacetamide was weakly cytotoxic and was not genotoxic. These results are difficult to explain by S_N2 reactivity alone but may involve the activation of dihaloacetamides by intracellular GSH or –SH compounds, which displace one halogen and form highly reactive α-halothioether electrophilic intermediates. The key element of this reaction is the presence of at least one halogen with good leaving tendency. With GSH-mediated activation, the weak activity of dichloroacetamide and similar combined toxicity indices between dibromoacetamide versus bromochloroacetamide may be expected (Table 7.3). The estimated log P values followed the order: I_2 > IBr > ICl > Br_2 > BrCl > Cl_2. This relative order is nearly identical to their cytotoxicity and genotoxicity. Log P may play a more important role in the activity of dihaloacetamides by affecting cellular uptake. The cytotoxicity and genotoxicity of trihaloacetamides decreased with a decrease in the number of bromo groups. The cytotoxicity of trichloroacetamide was lower than tribromoacetamide by nearly three orders of magnitude; this confirmed results in human leukemia P388 cells (Kigoshi et al. 2004). Only one bromo group was required for potent cytotoxicity; the %C½ values of tribromoacetamide, dibromochloroacetamide, and bromodichloroacetamide were within the same order of magnitude. In contrast, there was a greater difference in genotoxic potency values (Table 7.2). The cytotoxicity and genotoxicity of trihaloacetamides could be partially explained by

electrophilic reactivity at the carbonyl carbon as well as the possible release of electrophilic dihalocarbene intermediates (see discussion above). Alternatively, it is possible that reductive dehalogenation may yield cytotoxic free radicals; this pathway and the metabolic competency of the CHO cells have only been partially defined (McGregor et al. 1991). Glutathione S-transferase theta 1-1 (GSTT1-1) catalyzes preferential activation of brominated trihalomethanes to genotoxic intermediates (Geter et al. 2004; Ross and Pegram 2004, 2003); the possible role of GSTT1-1 in the activation of trihaloacetamides in CHO cells remains to be explored.

Table 7.3

Estimated log P values and combined Toxicity Index values of the haloacetamides studied

Compound	Estimated log P [a]	Combined Toxicity Index Value [b]
Monohaloacetamides		
Iodoacetamide	−0.08	5.63×10^{4}
Bromoacetamide	−0.49	5.17×10^{4}
Chloroacetamide	−0.58	1.31×10^{3}
Dihaloacetamides		
Diiodoacetamide	0.92	5.78×10^{4}
Dibromoacetamide	0.09	2.64×10^{3}
Dichloroacetamide	−0.09	1.68×10^{2}
Bromoiodoacetamide	0.50	1.02×10^{5} [c]
Chloroiodoacetamide	0.41	6.49×10^{3}
Bromochloroacetamide	0.00	3.33×10^{3}
Trihaloacetamides		
Tribromoacetamide	1.10	5.61×10^{4}
Dibromochloroacetamide	1.01	2.70×10^{4}
Bromodichloroacetamide	0.92	1.29×10^{4}
Trichloroacetamide	0.83	2.33×10^{2}

[a] Calculated using the KOWWIN program (version 1.67) developed by U.S. EPA using the atom/fragment approach (program available at http://www.epa.gov/oppt/newchems/pubs/sustainablefutures.htm).

[b] The Combined Toxicity Index is the reciprocal of the averaged %C½ and the SCGE genotoxic potency values. A larger Toxicity Index value indicates greater overall toxicity.

[c] Calculation based on the estimated bromoiodoacetamide values alone.

CHAPTER 8
HALOALDEHYDES

INTRODUCTION

The haloaldehydes evaluated in this study were chloroacetaldehyde, dibromoacetaldehyde, dichloroacetaldehyde, tribromoacetaldehyde, and trichloroacetaldehyde (Table 2.1).

OCCURRENCE

The aldehydes are toxic DBPs and several were measured in the ICR report, including formaldehyde, acetaldehyde, glyoxal, methylglyoxal, and trichloroacetaldehyde (chloral hydrate). Although the non-halogenated aldehydes are DBPs produced primarily by ozone treatment (Glaze et al. 1991; Richardson 1998), chlorine and chlorine dioxide treatment can form formaldehyde (Richardson 1998; McGuire, McLain, and Obolensky 2002; Richardson 2003). In the ICR, these aldehydes were detected at higher concentrations in water treatment systems using ozone (up to 30.6 µg/L) than plants using chlorine dioxide. Among treatment systems using ozone, the 90th percentile concentration for formaldehyde was 13.7 µg/L. Formaldehyde was found in over 50% of the water treatment plants using chlorine dioxide with a mean concentration of 5.3 µg/L and 90th percentile of 9.0 µg/L (McGuire, McLain, and Obolensky 2002). Acetaldehyde, glyoxal, and methyl glyoxal were observed at maximum levels of 11, 16, and 6 µg/L, respectively, in ozonated drinking water, but generally <5 µg/L in chlorine dioxide-treated waters. Chloral hydrate is a DBP associated with chlorine or chloramine treatment, but ozonation prior to chlorination or chloramination treatment can increase its formation in finished water (Krasner et al. 2006; McKnight and Reckhow 1992). Interestingly chloral hydrate was found at higher levels in the distribution system than in the finished water (McKnight and Reckhow 1992). Chloro-, dichloro-, bromochloro-, and tribromoacetaldehyde were included in the Nationwide Occurrence Study: the haloaldehydes were the third largest DBP class by weight (Krasner et al. 2006). Of the haloaldehydes studied, dichloroacetaldehyde was the most abundant with a maximum concentration of 16 µg/L. Ozonation followed by postchloramination was found to increase the formation of haloaldehydes.

GENOTOXICITY

The genotoxicity of formaldehyde was recently reviewed (IARC 2006). It required cytochrome P-450 mediated monooxygenation to be mutagenic *in vitro*, and induced gene mutation in bacteria, mammalian cells, and in rat nasal epithelia *in vivo*. It also induced sister chromatid exchanges (SCEs) in mammalian cells, as well as micronuclei and chromosomal aberrations in mammalian cells and rodents. It induced DNA damage in bacteria and mammalian cells and germ-cell mutations in *Drosophila* and possibly rodents. Formaldehyde generated DNA–protein cross-links in rodents and humans. In mouse lymphoma cells, formaldehyde induced gene mutations containing large deletions and recombination events (Speit and Schmid 2006; Speit and Merk 2002). The genotoxicity of acetaldehyde was reviewed (IARC 1999). It required S9 microsomes to be mutagenic *in vitro*; however, it was not mutagenic in bacteria. In mammalian cells, it caused gene mutations, SCEs, micronuclei, and chromosomal aberrations. In rodents, it induced

SCEs and protein-DNA cross-links. Acetaldehyde caused aneuploidy in fungi. The genotoxicity of chloral hydrate was reviewed recently (Moore and Harrington-Brock 2000; IARC 2004). Chloral hydrate is a direct-acting mutagen *in vitro* and induced base-substitution mutations in bacteria, aneuploidy and micronuclei in mammals *in vivo*, and aneuploidy, micronuclei, chromosomal aberrations, gene mutations, and cell transformation in mammalian cells *in vitro*. In addition it caused DNA damage and protein-DNA cross links in rodents. Chloral hydrate is metabolized in humans and rodents to trichloroacetic acid, trichloroethanol, and dichloroacetic acid (Moore and Harrington-Brock 2000). Chloroacetaldehyde is mutagenic in bacteria and mammalian cells *in vitro* (Bartsch, Camus, and Malaveille 1976) and is a metabolite of the well-characterized mutagen and carcinogen vinyl chloride (Chiang et al. 1997). Glyoxal is a related aldehyde that is mutagenic in bacteria (Jung et al. 1992; Muller et al. 1993; Murata-Kamiya, Kaji, and Kasai 1997; Murata-Kamiya et al. 1997) and it was suggested that glyoxal induced mutations via an oxygen radical mechanism. Glyoxal induced DNA strand breaks and DNA-protein cross-links in human skin cells (Kuchenmeister, Schmezer, and Engelhardt 1998; Roberts et al. 2003). The genotoxicity of methylglyoxal was reviewed (IARC 1991). It was a direct-acting mutagen in bacteria (Kasai et al. 1982; Nagao et al. 1986) and induced gene mutation, gene conversion, chromosomal aberrations, and micronuclei in yeast and mammalian cells (IARC 1991). Mutations induced in mammalian cells were mainly deletions and base substitutions (Murata-Kamiya and Kamiya 2001).

CHO CELL CYTOTOXICITY ANALYSIS OF THE HALOALDEHYDES

The CHO cell chronic cytotoxicity of the five haloaldehydes analyzed in this study are presented in Table 8.1. In the Table, the lowest concentration of a specific haloaldehyde 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 haloaldehyde 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.

Table 8.1
CHO cell chronic cytotoxicity of the haloaldehyde DBPs and related chemicals

Compound	Lowest Tox. Conc. (M)	R^2	%C½ (M)	ANOVA Test Statistic
Chloroacetaldehyde	5.00×10^{-7}	0.99	3.60×10^{-6}	$F_{11,\,176} = 240.7$; $P \leq 0.001$
Dibromoacetaldehyde	2.00×10^{-6}	0.99	4.70×10^{-6}	$F_{10,\,177} = 164.8$; $P \leq 0.001$
Dichloroacetaldehyde	8.00×10^{-6}	0.91	2.93×10^{-5}	$F_{19,\,328} = 36.1$; $P \leq 0.001$
Tribromoacetaldehyde	2.00×10^{-6}	0.99	3.58×10^{-6}	$F_{18,\,102} = 42.8$; $P \leq 0.001$
Trichloroacetaldehyde	3.75×10^{-4}	0.94	1.16×10^{-3}	$F_{24,\,333} = 33.9$; $P \leq 0.001$

The CHO cell chronic cytotoxicity concentration-response curves are presented for each haloaldehyde in the following figures: chloroacetaldehyde (Figure 8.1), dibromoacetaldehyde (Figure 8.2), dichloroacetaldehyde (Figure 8.3), tribromoacetaldehyde (Figure 8.4), and trichloroacetaldehyde (Figure 8.5).

The lowest concentration that induced a significant cytotoxic response ranged from 0.5 µM (chloroacetaldehyde) to 375 µM (trichloroacetaldehyde) (Table 8.1). The %C½ values ranged from 3.58 µM (tribromoacetaldehyde) to 1.16 mM (trichloroacetaldehyde). Based on the %C½ values the rank order for cytotoxicity was tribromoacetaldehyde ≈ chloroacetaldehyde > dibromoacetaldehyde > dichloroacetaldehyde >> trichloroacetaldehyde. A comparison of the relative cytotoxicity of the haloacetaldehydes analyzed in this study is presented in Figure 8.6.

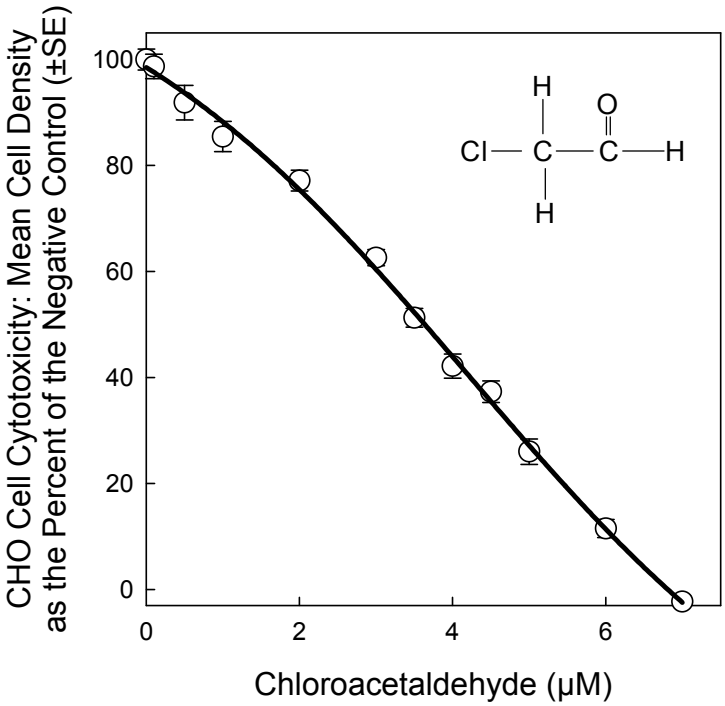


Figure 8.1 CHO cell chronic cytotoxicity concentration-response curve for chloroacetaldehyde

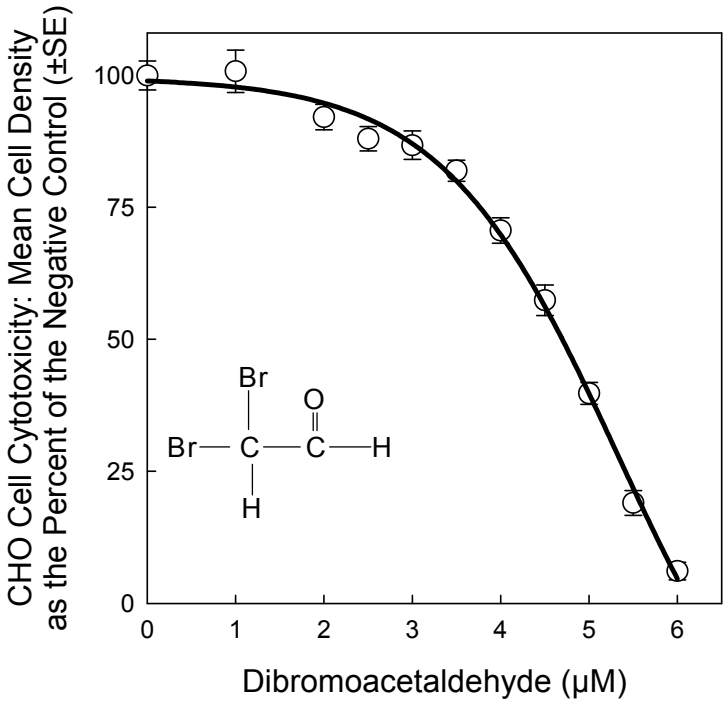


Figure 8.2 CHO cell chronic cytotoxicity concentration-response curve for dibromoacetaldehyde

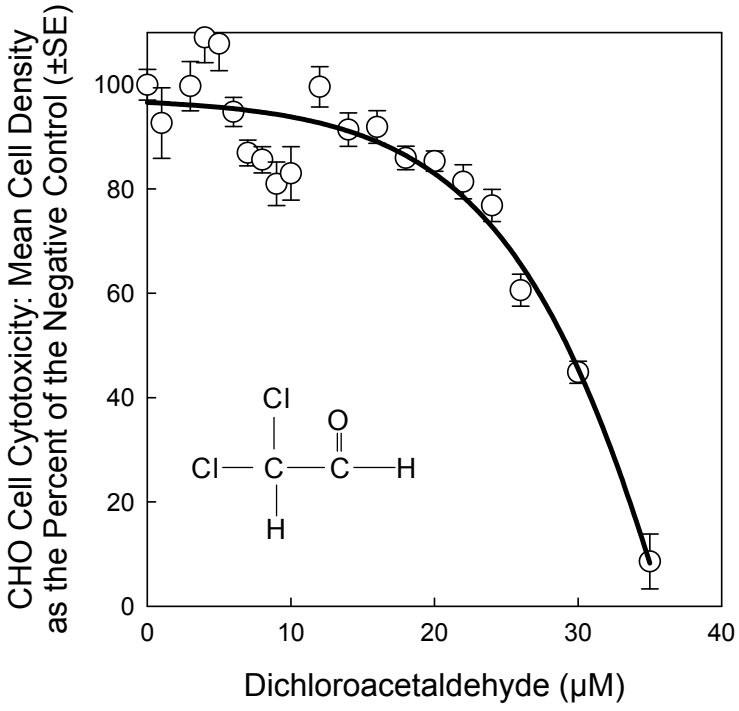


Figure 8.3 CHO cell chronic cytotoxicity concentration-response curve for dichloroacetaldehyde

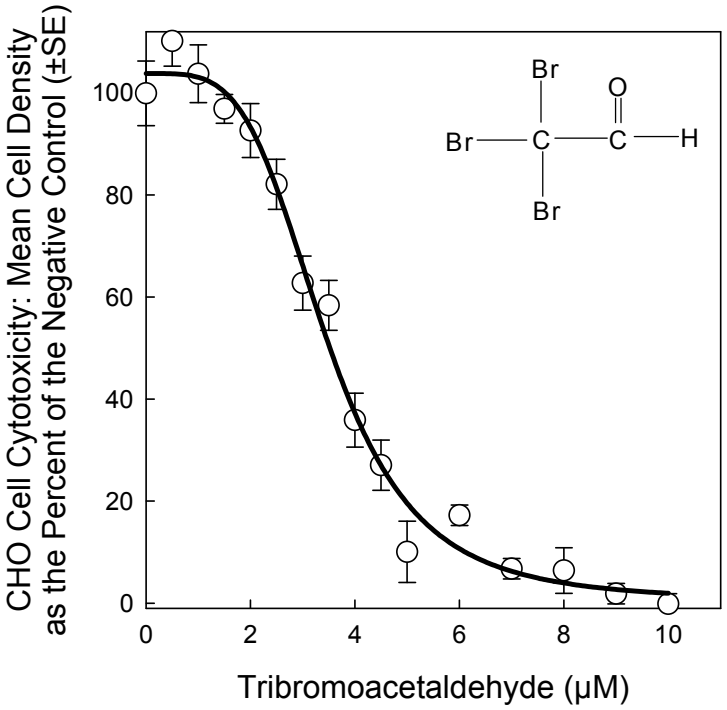


Figure 8.4 CHO cell chronic cytotoxicity concentration-response curve for tribromoacetaldehyde

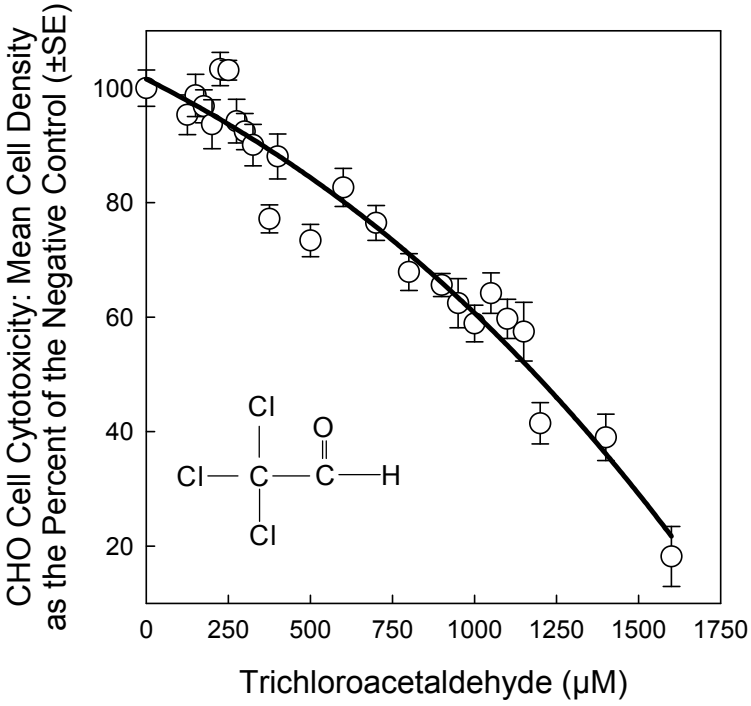


Figure 8.5 CHO cell chronic cytotoxicity concentration-response curve for trichloroacetaldehyde

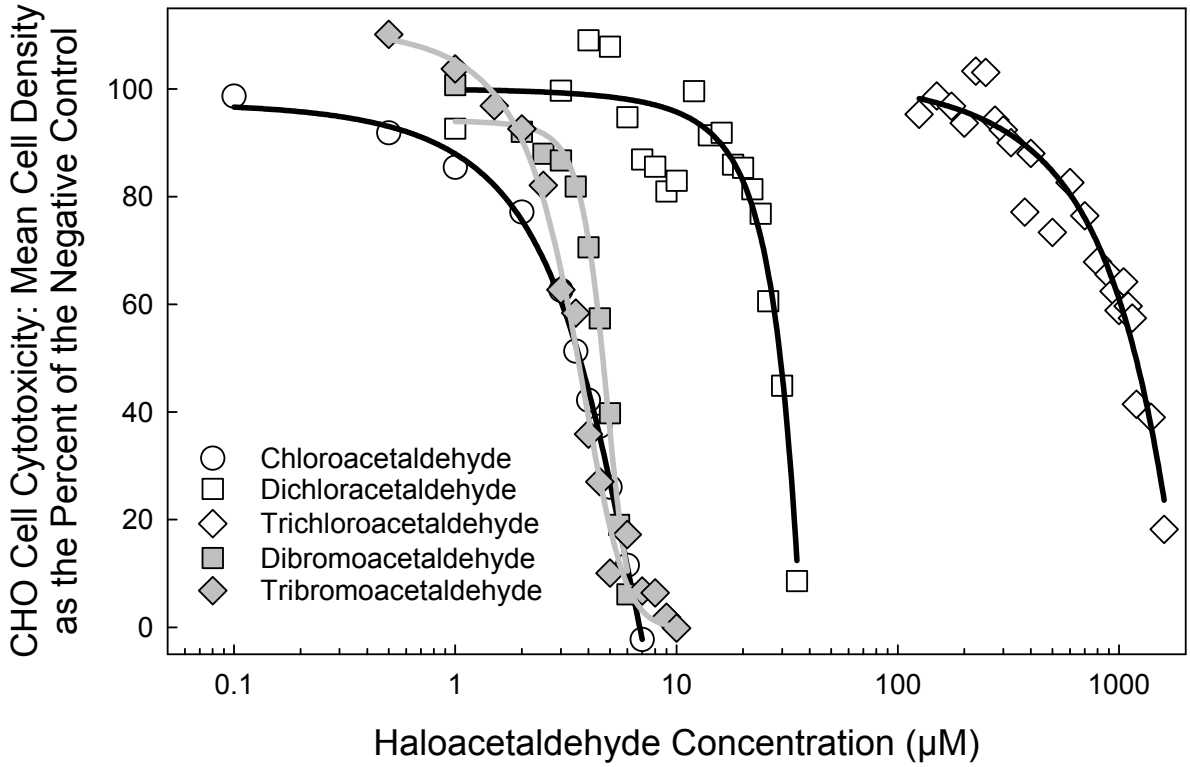


Figure 8.6 Comparison of the concentration-response curves for the CHO cell chronic cytotoxicity of the haloacetaldehydes

CHO CELL GENOTOXICITY ANALYSIS OF THE HALOACETALDEHYDES

In this study five haloacetaldehydes were analyzed for their ability to induce genomic DNA damage in CHO cells; the results are presented in Table 8.2. In the table, the lowest concentration of a specific haloacetaldehyde 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.

Table 8.2
CHO cell genotoxicity of the haloacetaldehyde DBPs and related chemicals

Compound	Lowest Genotox. Conc. (M)	R^2	SCGE Gen. Potency (M)	ANOVA Test Statistic
Chloroacetaldehyde	1.25×10^{-4}	0.98	1.59×10^{-4}	$F_{9,\,58} = 56.7$; $P \leq 0.001$
Dibromoacetaldehyde	1.00×10^{-4}	0.98	1.64×10^{-4}	$F_{8,\,43} = 55.7$; $P \leq 0.001$
Dichloroacetaldehyde	7.00×10^{-4}	0.96	8.83×10^{-4}	$F_{21,\,88} = 31.9$; $P \leq 0.001$
Tribromoacetaldehyde	2.50×10^{-4}	0.97	3.55×10^{-4}	$F_{11,\,64} = 70.99$; $P \leq 0.001$
Trichloroacetaldehyde	NS	–	NS	$F_{20,\,37} = 1.2$; $P = 0.29$

NS = not statistically different from the negative control.

The CHO cell concentration-response curves illustrating the induction of genomic DNA damage are presented for chloroacetaldehyde, dibromoacetaldehyde, dichloroacetaldehyde, tribromoacetaldehyde, and trichloroacetaldehyde in Figures 8.7 - 8.11, respectively. A comparison of the CHO cell genotoxicity for these agents is presented in Figure 8.12. The lowest concentration that induced a significant SCGE genotoxic response ranged from 100 µM for dibromoacetaldehyde to 700 µM for dichloroacetaldehyde. The SCGE genotoxic potency value ranged from 159 µM for chloroacetaldehyde to 883 µM for dichloroacetaldehyde (Table 8.2). The rank order of genotoxic potency from most to least potent was chloroacetaldehyde ≈ dibromoacetaldehyde > tribromoacetaldehyde > dichloroacetaldehyde. Trichloroacetaldehyde was not genotoxic (Figure 8.12).

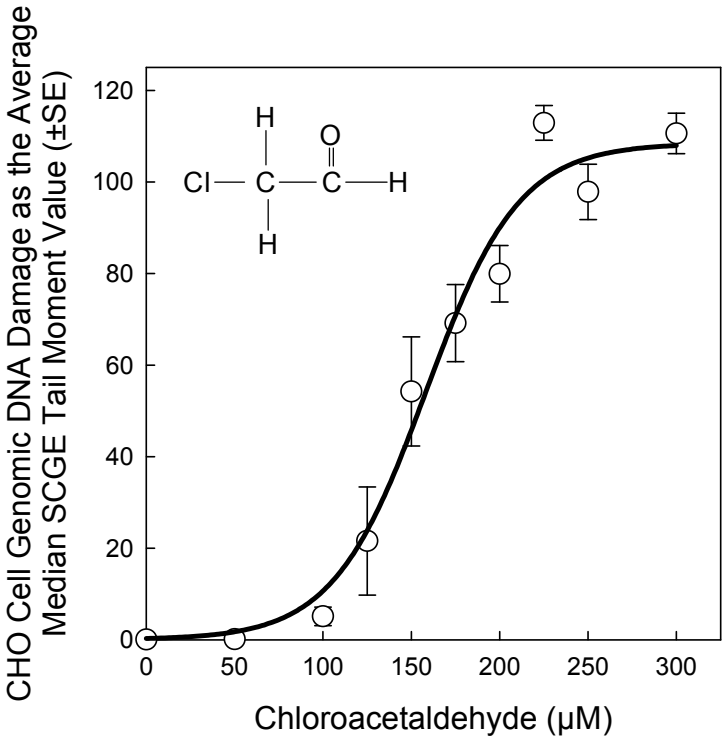


Figure 8.7 CHO cell SCGE genotoxicity concentration-response curve for chloroacetaldehyde

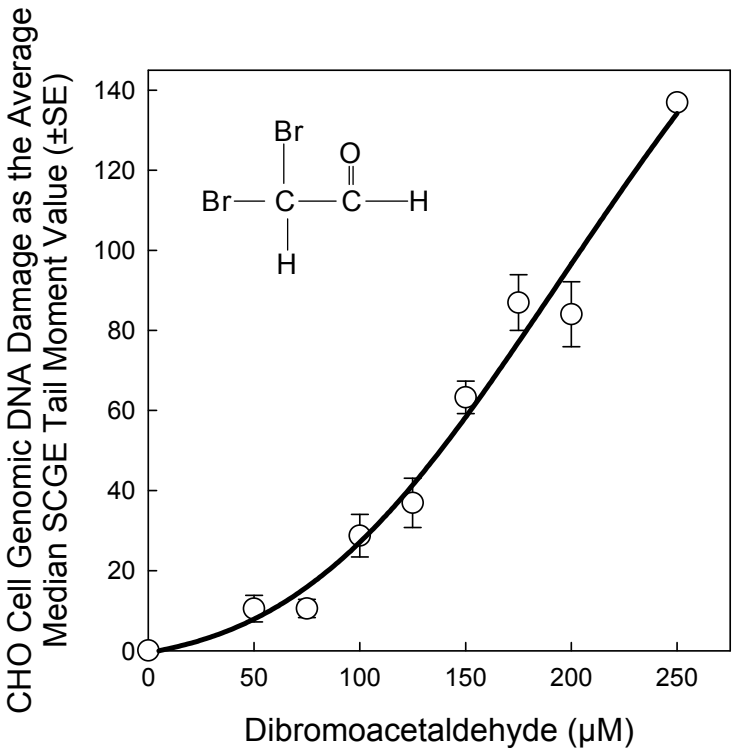


Figure 8.8 CHO cell SCGE genotoxicity concentration-response curve for dibromoacetaldehyde

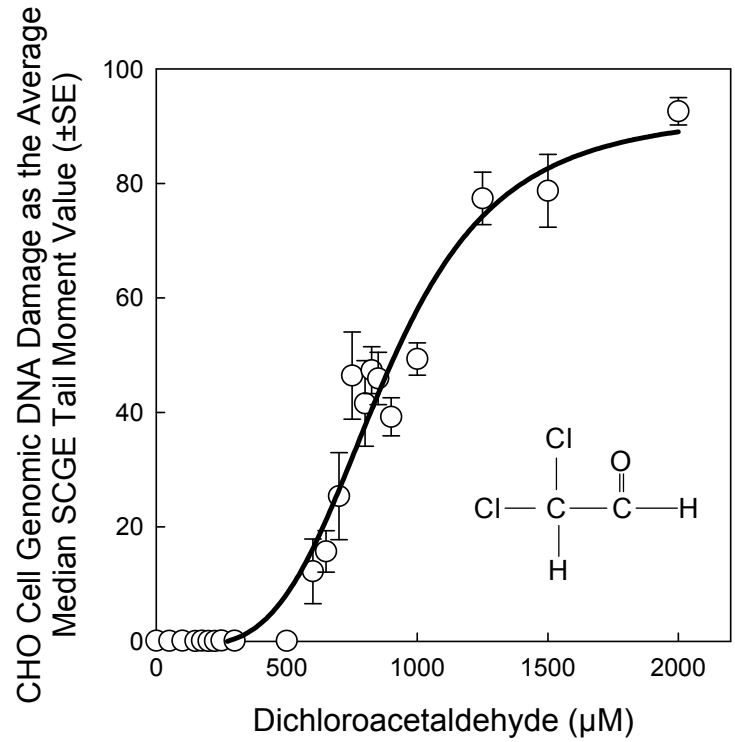


Figure 8.9 CHO cell SCGE genotoxicity concentration-response curve for dichloroacetaldehyde

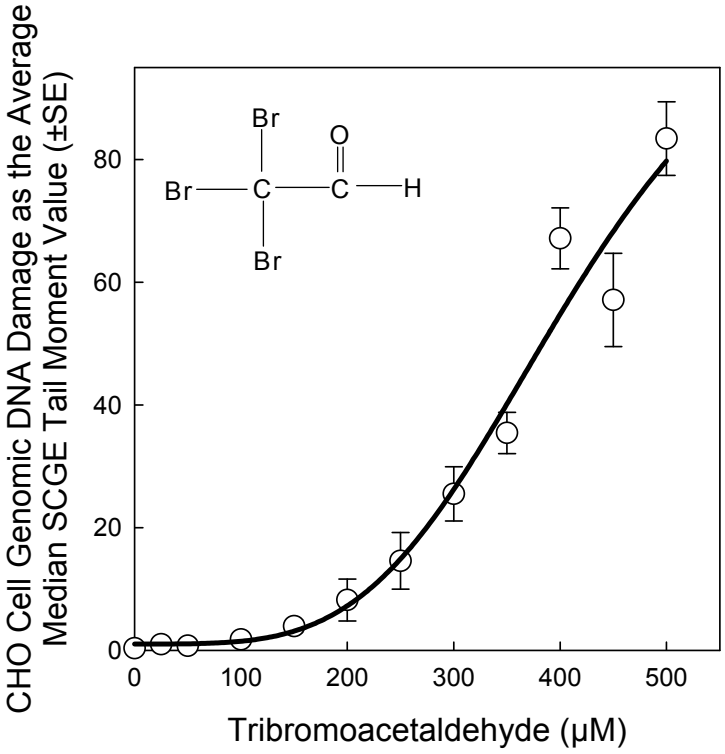


Figure 8.10 CHO cell SCGE genotoxicity concentration-response curve for tribromoacetaldehyde

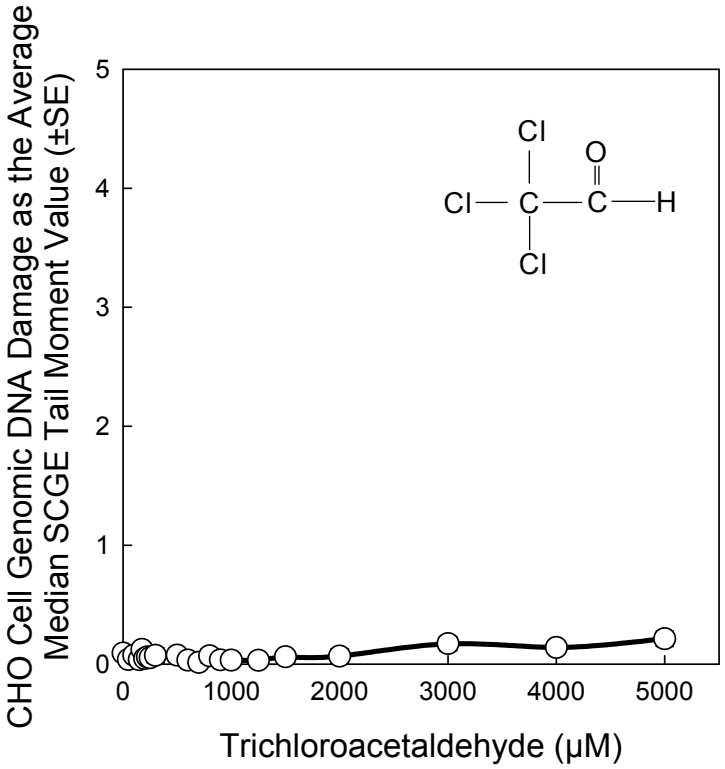


Figure 8.11 CHO cell SCGE genotoxicity concentration-response curve for trichloroacetaldehyde

Comparative CHO Cell Cytotoxicity and Genotoxicity of the Haloacetaldehydes

A comparison of the CHO cell chronic cytotoxicity of five haloacetaldehydes is presented in Figure 8.6. The rank order of the cytotoxicity for these agents is chloroacetaldehyde ≈ tribromoacetaldehyde > dibromoacetaldehyde > dichloroacetaldehyde >> trichloroacetaldehyde. For the chlorinated acetaldehydes there was a decrease in cytotoxicity with an increase in the number of halogen atoms. The brominated species expressed a departure from this pattern; tribromoacetaldehyde was slightly more cytotoxic than dibromoacetaldehyde. As illustrated in Figure 8.12, there was a decrease in genotoxicity with an increase in the number of bromine or chlorine atoms. As observed with other DBP classes and related compounds analyzed in this research, the brominated acetaldehydes were more cytotoxic and genotoxic than their corresponding chlorinated analogues.

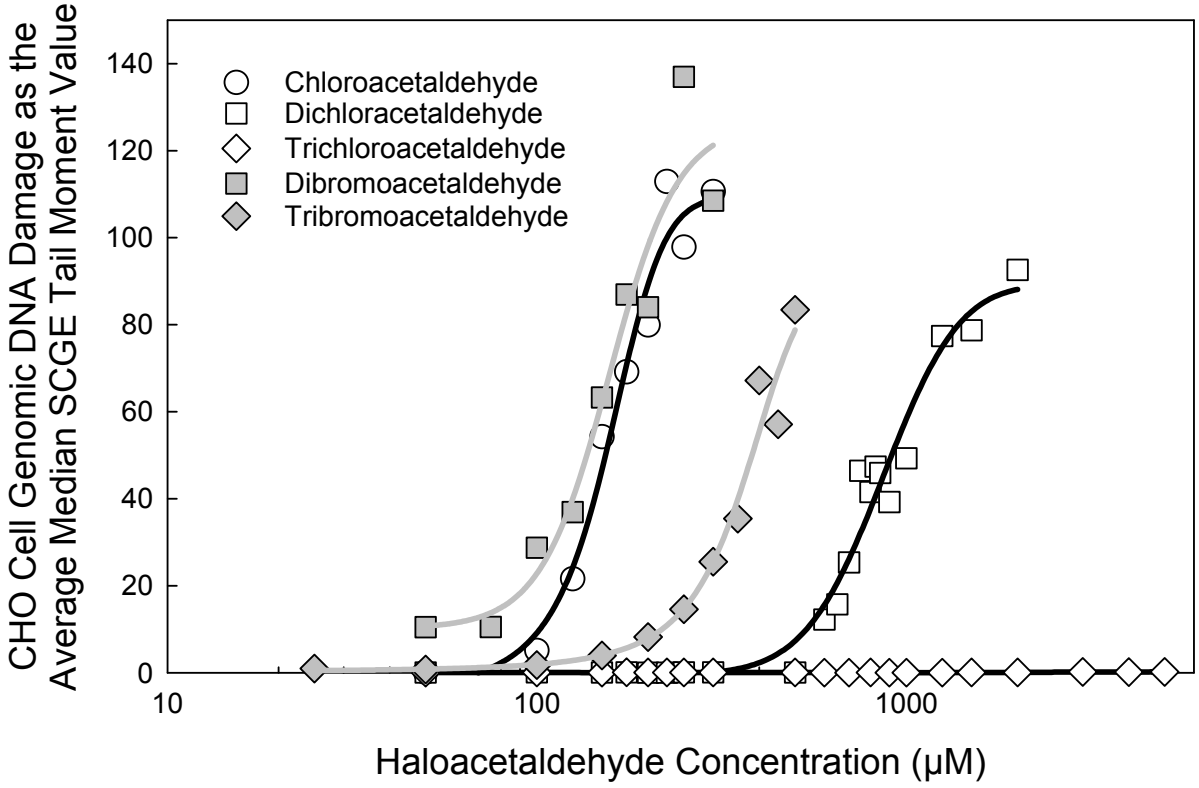


Figure 8.12 Comparison of the SCGE genotoxicity concentration-response curves of the haloacetaldehydes

CHAPTER 9
COMPARISON OF THE CHRONIC CYTOTOXICITY AND GENOTOXICITY OF DBP CLASSES

INTRODUCTION

In this project 47 DBPs or related compounds amongst six chemical classes were evaluated for their *in vitro* chronic cytotoxicity or acute genotoxicity in mammalian cells. These results represent the largest single systematic biological analysis of DBPs conducted to date. When combined with previously published and unpublished data, this *in vitro* CHO cell analysis has generated a large database of over 60 DBPs systematically analyzed. A summary of the CHO cell chronic cytotoxicity (as %C½ values) and the acute genotoxicity (as SCGE genotoxic potency values) is presented in Table 9.1. The DBPs and related compounds are presented by chemical class and the compounds are listed in alphabetical order within each class.

Table 9.1
Summary of the CHO cell chronic cytotoxicity (%C½ value) and acute genotoxicity (SCGE potency value) of DBPs and related chemical agents

DBP or Chemical Agent	Chemical Class	%C½ Value (M)	SCGE Potency Value (M)
Bromochloroiodomethane	Halomethane	2.42×10^{-3}	NS
Bromodichloromethane	Halomethane	1.15×10^{-2}	NS
Chlorodibromomethane	Halomethane	5.36×10^{-3}	NS
Dibromoiodomethane	Halomethane	1.91×10^{-3}	NS
Tribromomethane (bromoform)	Halomethane	3.96×10^{-3}	NS
Trichloromethane (chloroform)	Halomethane	9.62×10^{-3}	NS
Triiodomethane (iodoform)	Halomethane	6.60×10^{-5}	NS
Bromochloroacetic acid	Haloacetic acid	7.78×10^{-4}	3.64×10^{-3}
Bromodichloroacetic acid	Haloacetic acid	6.85×10^{-4}	NS
Bromoiodoacetic acid	Haloacetic acid	8.97×10^{-4}	3.16×10^{-3}
Chlorodibromoacetic acid	Haloacetic acid	2.02×10^{-4}	1.36×10^{-2}
Diiodoacetic acid	Haloacetic acid	3.32×10^{-4}	1.98×10^{-3}
3-Bromo-3-iodopropenoic acid	Haloacid	1.89×10^{-4}	NS
2,3-Dibromopropenoic acid	Haloacid	2.20×10^{-3}	7.85×10^{-3}
3,3-Dibromopropenoic acid	Haloacid	2.95×10^{-4}	NS
2-Iodo-3-bromopropenoic acid	Haloacid	4.36×10^{-5}	7.58×10^{-3}
2,3,3-Tribromopropenoic acid	Haloacid	1.64×10^{-3}	NS
3-Bromo-3-chloro-4-oxopentanoic acid	Haloacid	2.89×10^{-5}	3.58×10^{-4}
3,3-Dibromo-4-oxopentanoic acid	Haloacid	1.64×10^{-5}	9.03×10^{-5}
2-Bromobutenedioic acid	Haloacid	2.06×10^{-3}	5.90×10^{-3}
(*E*)-2-Iodo-3-methylbutenedioic acid	Haloacid	9.44×10^{-4}	6.00×10^{-3}

(continued)

Table 9.1 (Continued)
Summary of the CHO cell chronic cytotoxicity (%C½ value) and acute genotoxicity (SCGE potency value) of DBPs and related chemical agents

DBP or Chemical Agent	Chemical Class	%C½ Value (M)	SCGE Potency Value (M)
trans-2-Bromo-3-methylbutenedioic acid	Haloacid	5.27×10^{-3}	NS
Bromoacetonitrile	Halonitrile	3.21×10^{-6}	3.85×10^{-5}
Bromochloroacetonitrile	Halonitrile	8.46×10^{-6}	3.24×10^{-4}
Chloroacetonitrile	Halonitrile	6.83×10^{-5}	6.01×10^{-4}
Dibromoacetonitrile	Halonitrile	2.85×10^{-6}	2.97×10^{-5}
Dichloroacetonitrile	Halonitrile	5.73×10^{-5}	2.75×10^{-3}
Iodoacetonitrile	Halonitrile	3.30×10^{-6}	3.71×10^{-5}
Trichloroacetonitrile	Halonitrile	1.60×10^{-4}	1.01×10^{-3}
Bromoacetamide	Haloamide	1.89×10^{-6}	3.68×10^{-5}
Bromochloroacetamide	Haloamide	1.71×10^{-5}	5.83×10^{-4}
Bromodichloroacetamide	Haloamide	8.68×10^{-6}	1.46×10^{-4}
Bromoiodoacetamide	Haloamide	3.81×10^{-6} [a]	7.21×10^{-5} [b]
Chloroacetamide	Haloamide	1.48×10^{-4}	1.38×10^{-3}
Chloroiodoacetamide	Haloamide	5.97×10^{-6}	3.02×10^{-4}
Dibromoacetamide	Haloamide	1.22×10^{-5}	7.44×10^{-4}
Dibromochloroacetamide	Haloamide	4.75×10^{-6}	6.94×10^{-5}
Dichloroacetamide	Haloamide	1.92×10^{-3}	NS
Diiodoacetamide	Haloamide	6.78×10^{-7}	3.39×10^{-5}
Iodoacetamide	Haloamide	1.42×10^{-6}	3.41×10^{-5}
Tribromoacetamide	Haloamide	3.14×10^{-6}	3.25×10^{-5}
Trichloroacetamide	Haloamide	2.05×10^{-3}	6.54×10^{-3}
Chloroacetaldehyde	Haloaldehyde	3.60×10^{-6}	1.59×10^{-4}
Dibromoacetaldehyde	Haloaldehyde	4.70×10^{-6}	1.64×10^{-4}
Dichloroacetaldehyde	Haloaldehyde	2.93×10^{-5}	8.83×10^{-4}
Tribromoacetaldehyde	Haloaldehyde	3.58×10^{-6}	3.55×10^{-4}
Trichloroacetaldehyde	Haloaldehyde	1.16×10^{-3}	NS

NS = not statistically significant from negative controls.
[a] The calculated %C½ value for bromoiodoacetamide alone assuming an additive model for the diiodoacetamide and dibromoacetamide contaminants was 3.35×10^{-6} M.
[b] 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.

COMPARATIVE CYTOTOXICITY AND GENOTOXICITY OF DBP CLASSES

Relationship Between Cytotoxicity and Genotoxicity

A large dataset was available for a Pearson Product Moment Correlation test (Box, Hunter, and Hunter 1978). This consisted of six chemical classes of DBPs and related compounds

from this study that induced significant chronic cytotoxicity and acute genotoxicity plus data published on the haloacetic acids (Plewa et al. 2002; Plewa, Wagner, Richardson et al. 2004) and the halonitromethanes (Plewa, Wagner, Jazwierska et al. 2004). For this entire dataset of 47 compounds, the relationship between the %C½ and the SCGE genotoxic potency values was analyzed (Figure 9.1). A direct, highly significant ($P \leq 0.001$) correlation was observed ($r = 0.58$). This correlation was independent of chemical class but limited to those agents that induced both a cytotoxic and genotoxic response.

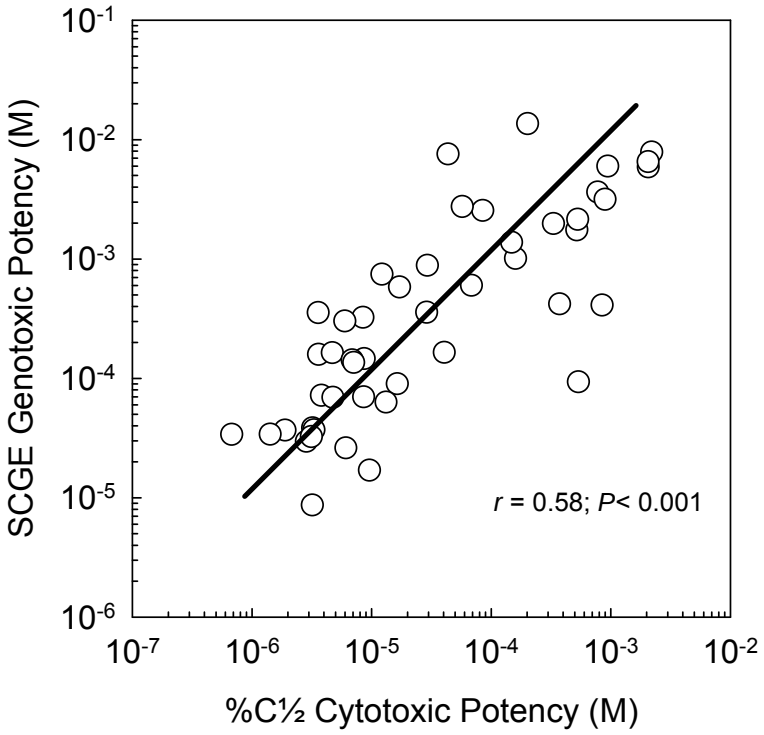


Figure 9.1 Correlation analyses between CHO cell chronic cytotoxicity and SCGE genotoxicity for 47 DBPs and related compounds

To determine if the relationship between CHO cell cytotoxicity and genotoxicity was universally expressed among the DBP chemical classes, a multiple correlation analysis was conducted. The results, presented in Table 9.2, indicate that the correlation was not applicable to all DBP chemical classes. The haloacetic acids, >2C-haloacids, and halonitriles did not exhibit a significant correlation between CHO cell chronic cytotoxicity and acute SCGE genotoxicity. The haloacetamides and the haloacetaldehydes were highly correlated ($P \leq 0.0001$ and $P \leq 0.05$, respectively). The halonitromethanes were correlated but were slightly below statistical significance ($P = 0.07$). These data indicate that the modes of biological action among these DBP classes differ considerably and that one cannot simply use one *in vitro* assay to establish their relative toxicity.

Table 9.2
Pearson Product Moment Correlation analysis of the CHO cell chronic cytotoxicity and acute SCGE genotoxicity among DBP chemical classes

DBP Chemical Class	*r*	*P*	Interpretation
Haloacetic Acids [a]	−0.04	0.91	no significant correlation
>2C-Haloacids	0.61	0.20	no significant correlation
Haloacetonitriles	0.45	0.31	no significant correlation
Haloacetamides	0.99	<0.0001	high, significant correlation
Haloacetaldehydes	0.96	<0.05	high, significant correlation
Halonitromethanes [a]	0.62	0.07	correlation

[a] In addition to the data generated by this project, data were from (Plewa, Wagner, Jazwierska et al. 2004; Plewa, Wagner, Richardson et al. 2004; Plewa et al. 2002).

Relative CHO Cell Chronic Cytotoxicity of DBPs and Related Compounds

The largest dataset for a systematic analysis of the *in vitro* CHO cell chronic cytotoxicity of DBPs and related compounds is illustrated in Figure 9.2. This figure represents 66 DBPs and related agents as well as a positive control, ethyl methanesulfonate. For each chemical the %C½ value was plotted (abscissa) and each chemical was organized as to chemical class (ordinate). The abbreviations used in this figure are: BAA = bromoacetic acid, BCAA = bromochloroacetic acid, BDCAA = bromodichloroacetic acid, BIAA = bromoiodoacetic acid, CAA = chloroacetic acid, CDBAA = chlorodibromoacetic acid, DBAA = dibromoacetic acid, DCAA = dichloroacetic acid, DIAA = diiodoacetic acid, IAA = iodoacetic acid, TBAA = tribromoacetic acid, TCAA = trichloroacetic acid, BNM = bromonitromethane, BCNM = bromochloronitromethane, BDCNM = bromodichloronitromethane, CNM = chloronitromethane, DBNM = dibromonitromethane, DBCNM = dibromochloronitromethane, DCNM = dichloronitromethane, TBNM = tribromonitromethane, TCNM = trichloronitromethane, MX = 3-chloro-4-(dichloromethyl)-5-hydroxy-2-(*5H*)-furanone, and EMS = ethyl methanesulfonate. The data presented in Figure 9.2 was generated by this study as well as from other studies using the CHO cell chronic cytotoxicity assay (Plewa, Muellner et al. 2008; Muellner et al. 2007; Stork et al. 2007; Cemeli et al. 2006; Plewa, Wagner, Jazwierska et al. 2004; Plewa, Wagner, Richardson et al. 2004; Richardson et al. 2003; Plewa et al. 2002, 2000). The value of this illustration is that over a molar concentration range of over 4 orders of magnitude, the relative CHO cell chronic cytotoxicity of each DBP can be compared to members within its chemical class or among DBPs from different chemical classes. Each %C½ value for each chemical was calculated from a concentration-response curve derived from approximately 180 – 240 individual cell cultures. Figure 9.2 also provides a comparison of the mammalian cell cytotoxicity among the U.S. EPA regulated DBPs versus emerging, unregulated DBPs and their related compounds. The most cytotoxic agent was diiodoacetamide while the least toxic was bromodichloromethane.

Relative CHO Cell Acute Genotoxicity of DBPs and Related Compounds

The largest dataset for a systematic analysis of the *in vitro* CHO cell SCGE genotoxicity of DBPs and related compounds is illustrated in Figure 9.3. Of the 66 DBPs and related agents listed in the figure, 75.8% induced significant levels of genomic DNA damage in CHO cells and 24.2% were not genotoxic. The well known mutagen ethyl methanesulfonate was employed as

the positive control. For each chemical the SCGE genotoxic potency value was plotted (abscissa) and each chemical was organized as to chemical class (ordinate). The abbreviations in this figure are: BAA = bromoacetic acid, BCAA = bromochloroacetic acid, BDCAA = bromodichloroacetic acid, BIAA = bromoiodoacetic acid, CAA = chloroacetic acid, CDBAA = chlorodibromoacetic acid, DBAA = dibromoacetic acid, DCAA = dichloroacetic acid, DIAA = diiodoacetic acid, IAA = iodoacetic acid, TBAA = tribromoacetic acid, TCAA = trichloroacetic acid, BNM = bromonitromethane, BCNM = bromochloronitromethane, BDCNM = bromodichloronitromethane, CNM = chloronitromethane, DBNM = dibromonitromethane, DBCNM = dibromochloronitromethane, DCNM = dichloronitromethane, TBNM = tribromonitromethane, TCNM = trichloronitromethane, MX = 3-chloro-4-(dichloromethyl)-5-hydroxy-2-(*5H*)-furanone, and EMS = ethyl methanesulfonate. The data presented in Figure 9.3 were generated by this study as well as from other studies using the CHO cell SCGE genotoxicity assay (Plewa, Muellner et al. 2008; Muellner et al. 2007; Stork et al. 2007; Cemeli et al. 2006; Plewa, Wagner, Jazwierska et al. 2004; Plewa, Wagner, Richardson et al. 2004; Richardson et al. 2003; Plewa et al. 2002, 2000) as well as unpublished data (Plewa, 2008). The value of this illustration is that over a molar concentration range of over 3 orders of magnitude, the relative CHO cell genomic DNA damaging capacity of each DBP can be compared to members within its chemical class or among DBPs from different chemical classes. Each SCGE genotoxic potency value for each chemical was calculated from a concentration-response curve derived from in general 8 concentrations with 6 replicate microgels per concentration. Of the DBPs that were genotoxic, the most potent was iodoacetic acid and the least potent was chlorodibromoacetic acid.

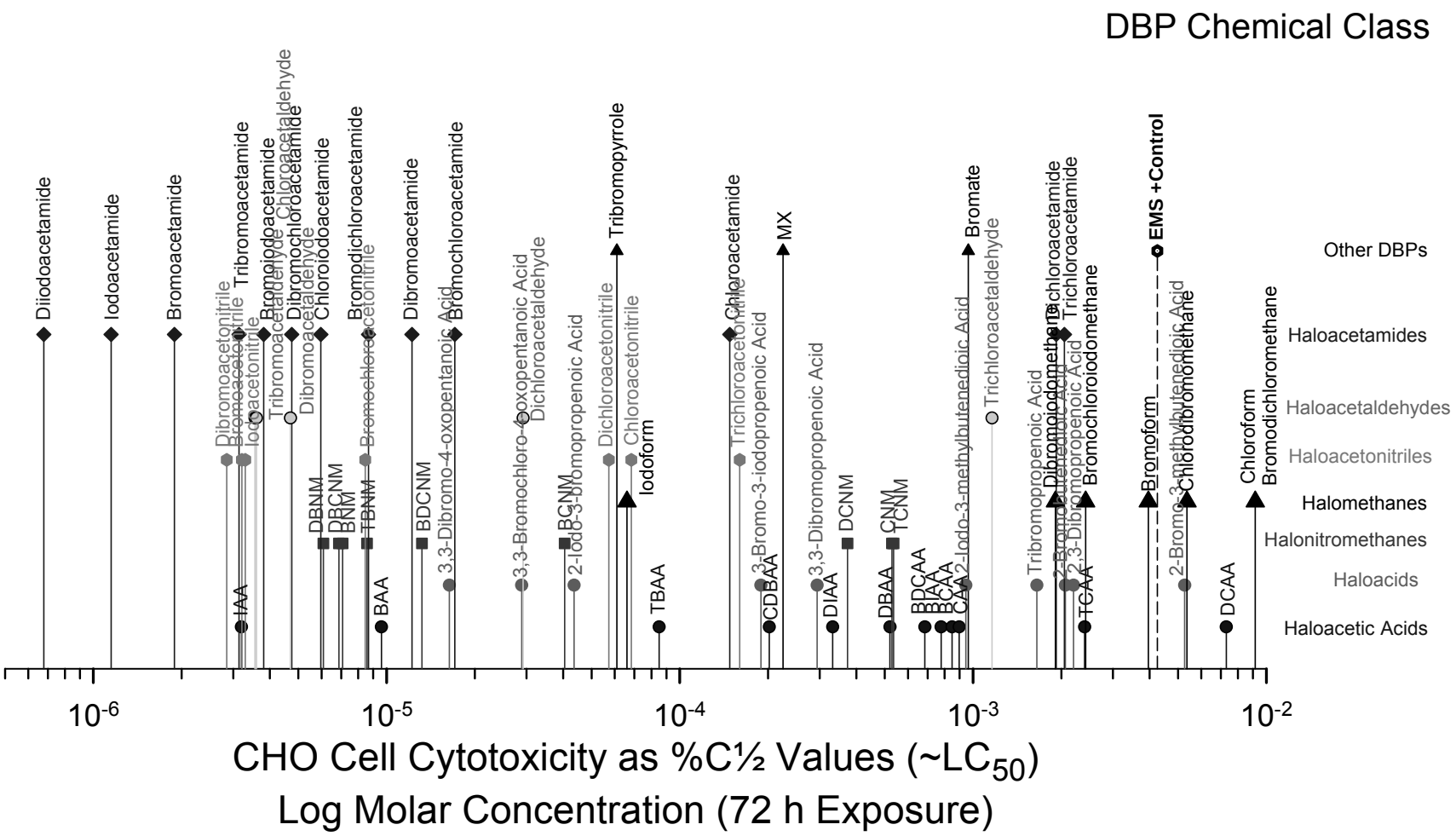
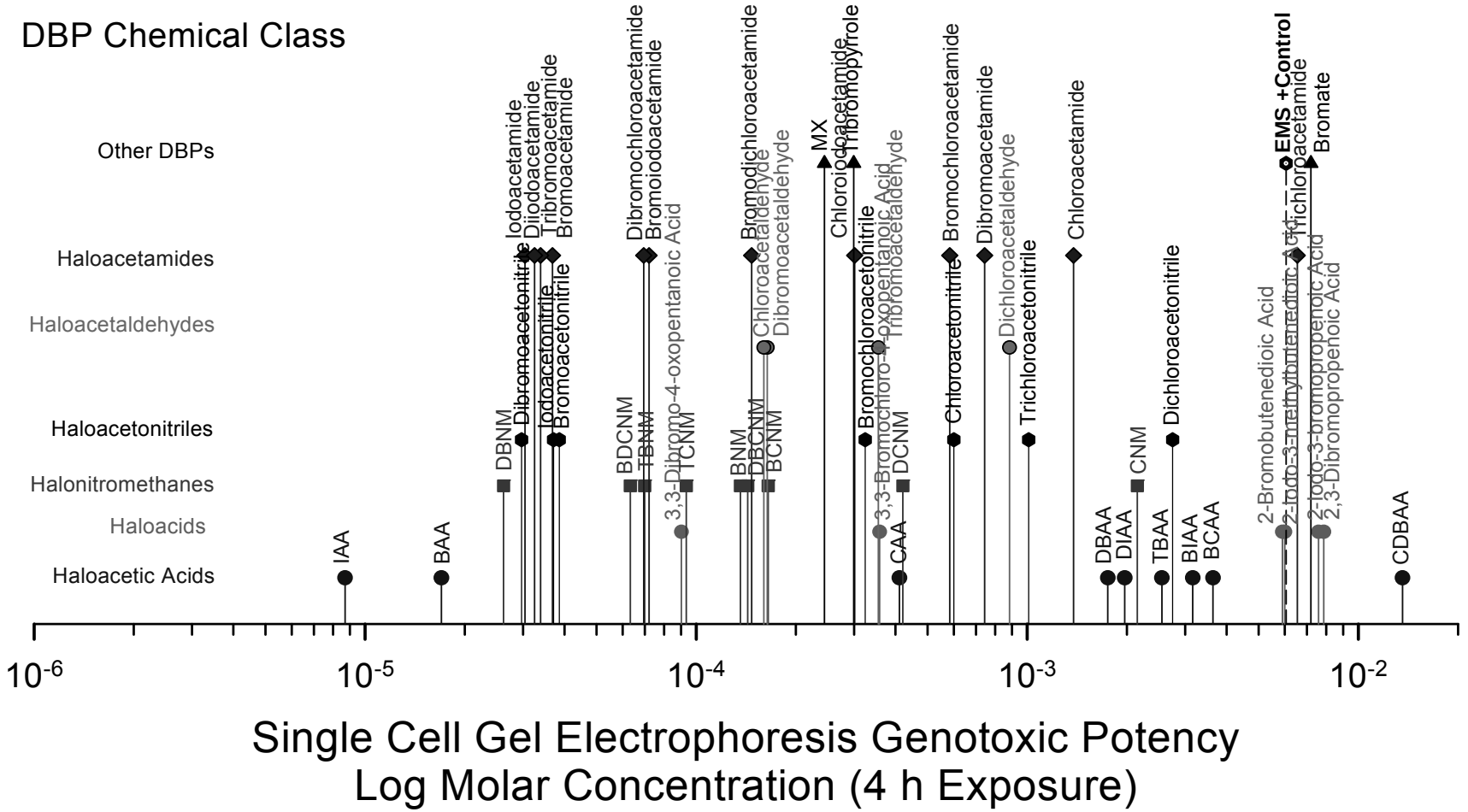


Figure 9.2 Comparative CHO cell chronic cytotoxicity induced by DBPs or related compounds



Not Genotoxic: DCAA, TCAA, BDCAA, Dichloroacetamide, Chloroform, Chlorodibromomethane, Bromoform, Iodoform, Bromochloroiodomethane, Dibromoiodomethane, Bromodichloromethane, 3,3-Dibromopropenoic Acid, 3-Bromo-3-iodopropenoic Acid, 2,3,3,Tribromopropenoic Acid, *trans*-2-Bromo-3-methylbutenedioic Acid, Trichloroacetaldehyde

Figure 9.3 Comparative CHO cell SCGE genotoxicity induced by DBPs or related compounds

Cytotoxicity and Genotoxicity Indices

Figures 9.2 and 9.3 provide a direct comparison of the relative cytotoxic and genotoxic potencies of DBPs and related compounds within their chemical class. From these two figures one can derive a qualitative overall toxic impact at the level of the chemical class, however it is difficult to quantitatively compare among DBP chemical classes. This becomes important when evaluating water utility changes in chemical and physical disinfection methods especially if alternatives to chlorine cause a modulation in the relative levels of specific DBP chemical classes. In order to generate a metric to compare relative toxicity at the level of DBP chemical classes, the CHO cell chronic cytotoxicity and acute genotoxicity indices were calculated. These indices allow a uniform comparison among groups of DBPs and one can ascertain their relative cytotoxicity or genotoxicity. The cytotoxicity index was determined by calculating the median %C½ value of all of the individual members within a single class of DBPs. The reciprocal was taken of this number so that a larger value was equated with that of higher cytotoxic potency. The genotoxicity index was determined by calculating the median SCGE genotoxic potency value from the individual members within a single class of DBPs. The reciprocal was taken of this number so that a larger value was equated with that of higher genotoxicity. In order to include compounds that were not genotoxic, a value of 1×10^{-2} M was used in the calculation. The cytotoxicity and genotoxicity indices for the classes of DBPs analyzed in this study with additional information on the haloacetic acids (Stork et al. 2007; Plewa et al. 2002) and the halonitromethanes (Plewa, Wagner, Jazwierska et al. 2004) are presented in Figure 9.4. These broader comparisons among DBP classes are possible because these agents were analyzed using identical assays, and all of the concentrations are in molar units. This figure was not adjusted for occurrence or the concentration of individual DBPs in drinking water which was not part of the objectives of this project. However, it is apparent that in general the DBP classes that are currently regulated (primarily the halomethanes and haloacetic acids) express the lowest cytotoxicity and genotoxicity index values while emerging, priority DBP classes show higher levels of both mammalian cell cytotoxicity and genotoxicity.

Comparison of the Cytotoxicity and Genotoxicity Indices of C-DBPs and N-DBPs

Using the database generated by the CHO cell assays, a comparison of the CHO cell cytotoxicity and genotoxicity indices for carbon-based DBPs (C-DBPs) versus nitrogen-containing DBPs (N-DBPs) is presented in Figure 9.5. At the present time there are no U.S. EPA regulated N-DBPs. The current move away from chlorine to alternative disinfectants and the use of contaminated or impaired source waters may alter the spectrum of DBPs to include many more emerging N-DBPs. The difference in their relative toxicities argues that additional attention should be focused on the N-DBPs and their possible adverse impacts on the public health and the environment (Plewa, Muellner et al. 2008; Muellner et al. 2007; Cemeli et al. 2006; Plewa, Wagner, Jazwierska et al. 2004; Plewa et al. 2002).

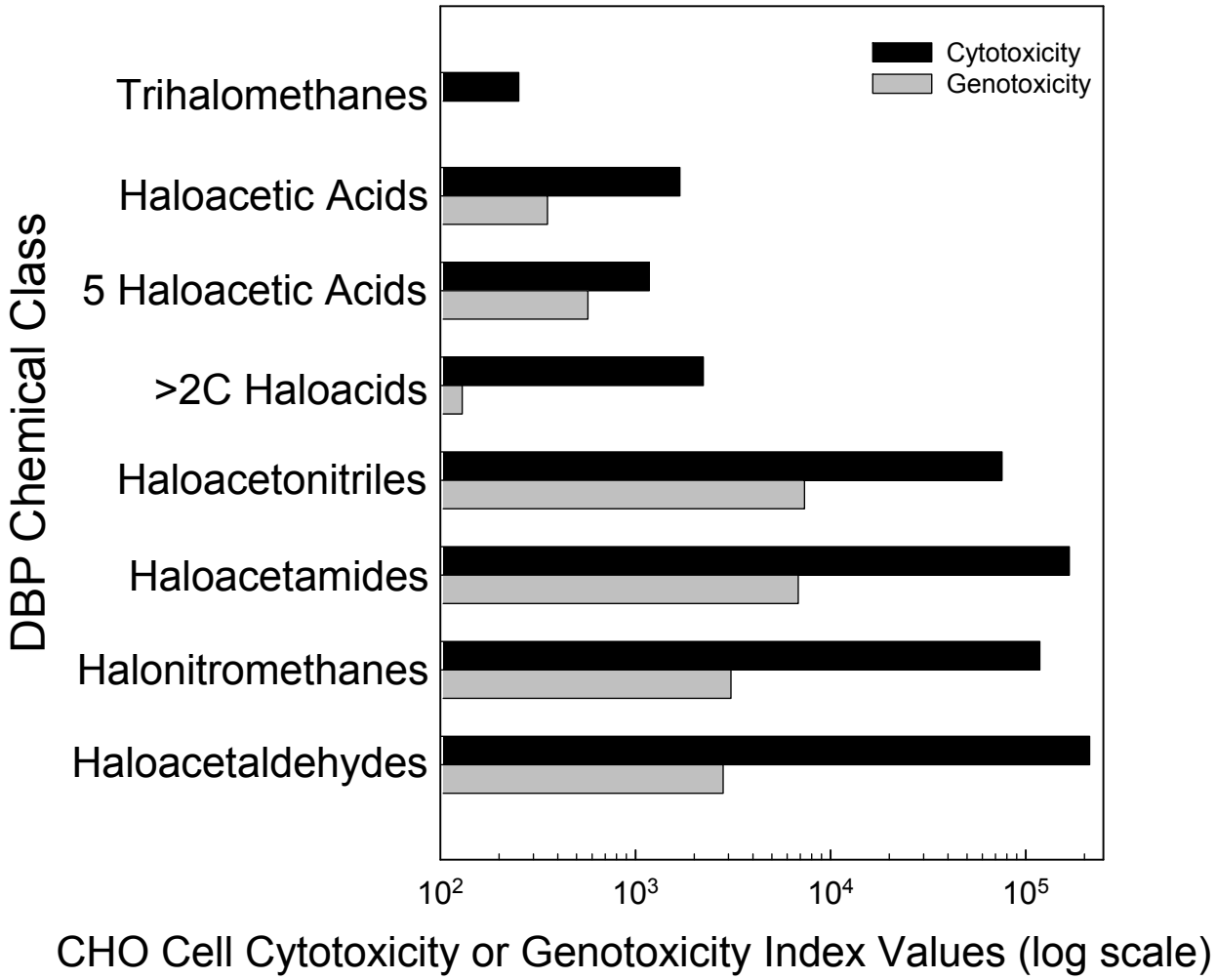


Figure 9.4 CHO cell cytotoxicity and genotoxicity indices for the major DBP chemical classes

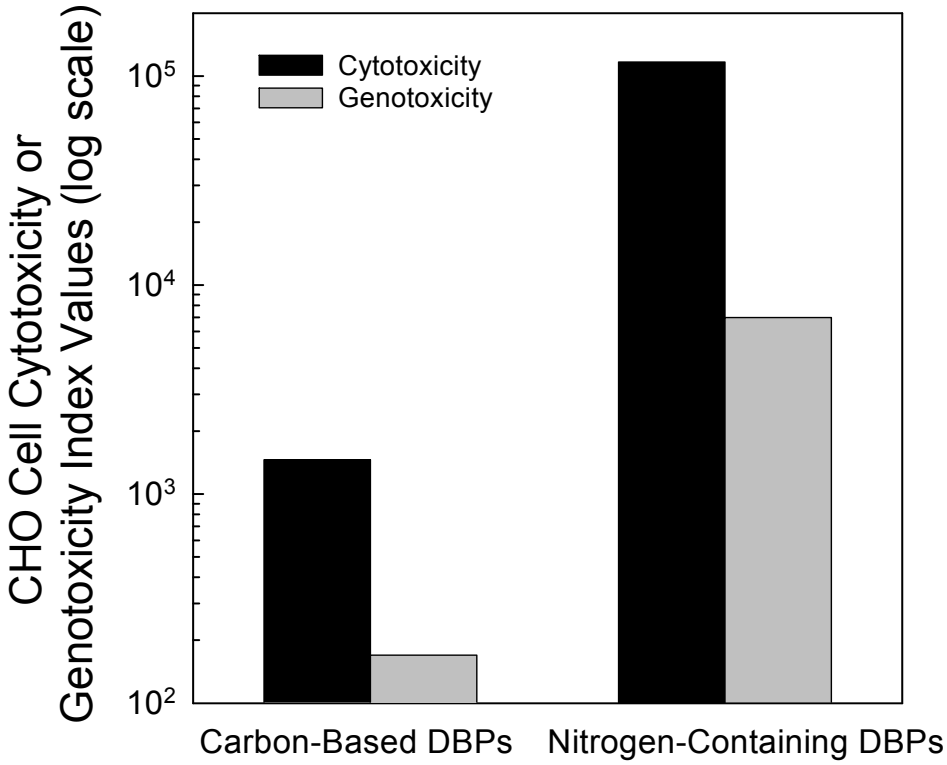


Figure 9.5 Cytotoxicity and genotoxicity indices for carbon-based DBPs (C-DBPs) versus nitrogen-containing DBPs (N-DBPs)

Impact of the Halogen Species on the DBP Toxicity Indices

The species of the halogen atom of specific DBPs generated a difference in cytotoxicity and genotoxicity in CHO cells. The rank order of decreasing DBP cytotoxicity and genotoxicity was iodo- > bromo- >> chloro-. The cytotoxicity and genotoxicity indices were individually calculated for bromo-, dibromo-, chloro-, dichloro, iodo- and diiodo acetic acids and acetamides, bromo-, chloro-, and iodoacetonitrile and chloroform, bromoform and iodoform. As illustrated in Figure 9.6 when a balanced design of these representative DBPs was analyzed, the iodinated DBPs were substantially more toxic than their brominated and chlorinated analogues. The data for this figure were from this study as well as from the published literature (Plewa, Muellner et al. 2008; Muellner et al. 2007; Stork et al. 2007; Cemeli et al. 2006; Richardson et al. 2006; Richardson et al. 2005; Plewa, Wagner, Jazwierska et al. 2004; Plewa, Wagner, Richardson et al. 2004; Richardson et al. 2003; Plewa et al. 2002, 2000). These data are important in that there are increasing numbers of treatment plants using chloramines to meet the U.S. EPA Stage 2 D/DBP Rule (U. S. Environmental Protection Agency 2006), and there may be increased occurrence of iodo-DBPs in those drinking waters (Richardson et al. 2007; Krasner et al. 2006; Richardson et al. 2006).

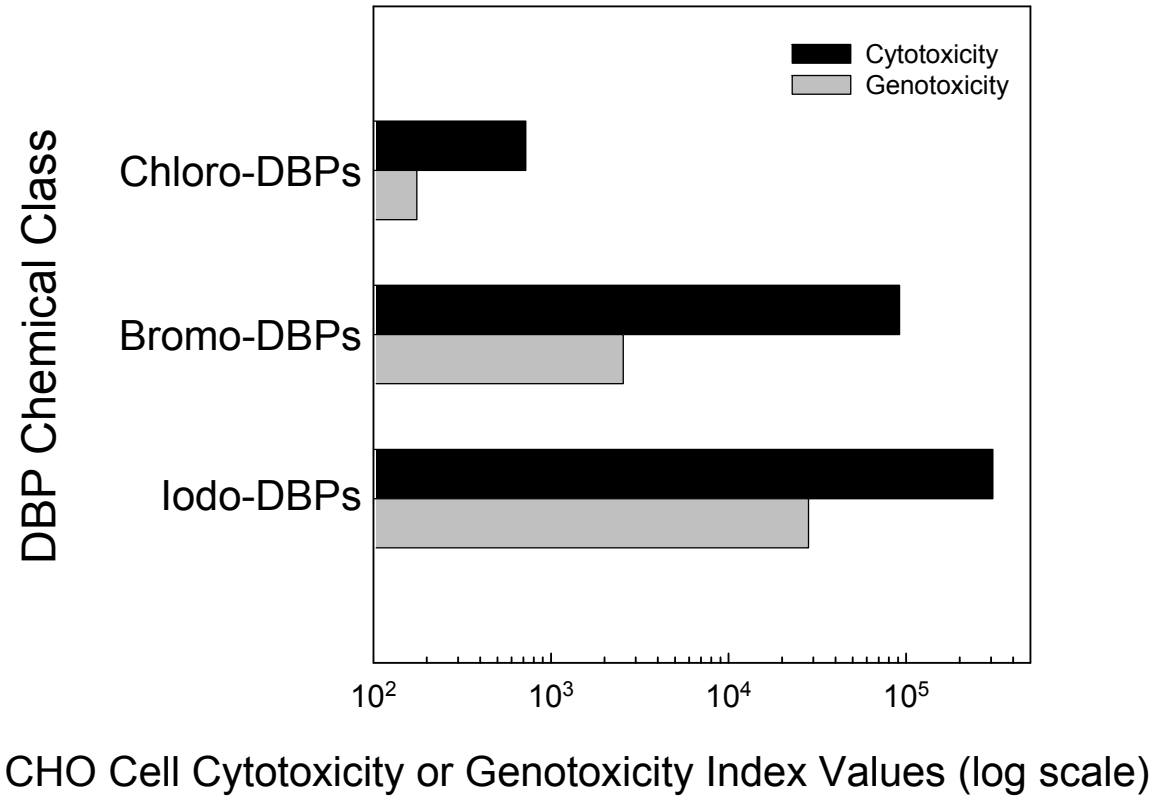


Figure 9.6 Cytotoxicity and genotoxicity indices of mono-, di- and trihalogenated DBPs and related compounds from the CHO cell database (6 compounds in each group)

CHAPTER 10
CONCLUSIONS

CONCLUSIONS

1. Forty-seven compounds from six DBP chemical classes that included halomethanes, haloacetic acids, >2C-haloacids, haloacetonitriles, haloacetamides and haloacetaldehydes were systematically analyzed for their *in vitro* chronic cytotoxicity and acute genotoxicity in mammalian cell assays. Of the 47 compounds analyzed, only 4 are currently regulated by the U.S. EPA.
2. The CHO cell cytotoxicity and genotoxicity results, when added to other published data constituted the largest comparative database of 66 DBPs and related agents.
3. The CHO cell chronic cytotoxicity of the DBPs encompassed concentrations over 4 log orders of magnitude with diiodoacetamide the most cytotoxic agent and bromodichloromethane the least cytotoxic.
4. The CHO cell genotoxicity of the DBPs encompassed concentrations over 3 orders of magnitude. A majority (75.8%) induced significant levels of genomic DNA damage. In this group iodoacetic acid was the most genotoxic; the least genotoxic was chlorodibromoacetic acid.
5. For CHO cell cytotoxicity, the rank order from most cytotoxic to least cytotoxic for the DBP classes was haloacetaldehydes > haloacetamides > halonitromethanes > haloacetonitriles > >2C-haloacids > haloacetic acids > halomethanes.
6. For induced genomic DNA damage in CHO cells, the rank order from the most genotoxic to the least genotoxic of the DBP classes was haloacetonitriles > haloacetamides > halonitromethanes > haloacetaldehydes > haloacetic acids > >2C-haloacids > halomethanes.
7. Although there was an overall significant correlation between chronic cytotoxicity and acute genotoxicity, this correlation did not apply to all DBP chemical classes.
8. Within an individual chemical class, there was a correlation between chronic cytotoxicity and acute genotoxicity for the haloacetamides, haloacetaldehydes, and to a lesser degree the halonitromethanes. The haloacetic acids, >2C-haloacids and the haloacetonitriles showed no such correlation.
9. In a balanced comparison of iodinated, brominated and chlorinated DBPs, the cytotoxicity and genotoxicity of the iodinated DBPs was greater than that of their brominated or chlorinated analogues with chlorinated analogues the least toxic.
10. Nitrogen-containing DBPs and related agents, including haloacetonitriles, haloacetamides, and halonitromethanes were far more cytotoxic and genotoxic than DBPs that did not contain nitrogen (haloacids, halomethanes and haloacetaldehydes).
11. These results are very relevant during the evaluation period for the U.S. EPA Stage 2 D/DBP Rule. 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.

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ABBREVIATIONS

α	alpha value is the acceptable probability of incorrectly rejecting the null hypothesis
ANOVA	analysis of variance
AS52	a transgenic clone of CHO cells derived from line K1 BH4
AWWA	American Water Works Association
AwwaRF	Awwa Research Foundation
BAA	bromoacetic acid
BAcAm	bromoacetamide
BAN	bromoacetonitrile
BCAA	bromochloroacetic acid
BCAcAm	bromochloroacetamide
BCAN	bromochloroacetonitrile
BCIM	bromochloroiodomethane
BCNM	bromochloronitromethane
BDCAA	bromodichloroacetic acid
BDCAcAm	bromodichloroacetamide
BDCM	bromodichloromethane
BDCNM	bromodichloronitromethane
BF	bromoform (tribromomethane)
BIAA	bromoiodoacetic acid
BIAcAm	bromoiodoacetamide
BNM	bromonitromethane
°C	degrees Celsius
>2C-haloacids	haloacids with more than 2 carbon atoms
CAA	chloroacetic acid
CAcAm	cloroacetamide
CAN	chloroacetonitrile
CDBAA	chlorodibromoacetic acid
CDBM	chlorodibromomethane
C-DBP	carbon-based DBP
CF	chloroform (trichloromethane)
CHO	Chinese hamster ovary cells
CIAcAm	chloroiodoacetamide
CNM	chloronitromethane
DBAA	dibromoacetic acid
DBAcAm	dibromoacetamide
DBAN	dibromoacetonitrile
DBCAcAm	dibromochloroacetamide
DBCNM	dibromochloronitromethane
DBIM	dibromoiodomethane

DBNM	dibromonitromethane
DBP	disinfection by-product
DCAA	dichloroacetic acid
DCAcAm	dichloroacetamide
DCAN	dichloroacetonitrile
DCNM	dichloronitromethane
DIAA	diiodoacetic acid
DIAcAm	diiodoacetamide
DMN-DM	dimethylnitrosamine-demethylase
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid disodium salt dehydrate
EI	electron ionization
*E*LUMO	lowest unoccupied molecular orbital
EMS	ethylmethanesulfonate
ex vivo	tissue or organ from a living organism
F12	Hams F12 medium
F value	the ratio of population of variances of normal populations
FBS	fetal bovine serum
g	gram
GC	gas chromatography
GSH	glutathione
GSTT1-1	glutathione-*S*-transferase-theta1-1
h	hour
HAN	haloacetonitriles
HAN4	the haloacetonitriles measured in the ICR
HBSS	Hank's balanced salt solution
IAA	iodoacetic acid
IAcAm	iodoacetamide
IARC	International Agency for Research on Cancer
ICR	U.S. EPA's Information Collection Rule
i.d.	internal diameter
in vitro	in an artificial environment outside the living organism
in vivo	within a living organism
IF	iodoform (triiodomethane)
KV	kilovolt
L	liter
log *P*	octanol-water partition coefficient
M	molar

m	meter
MCL	maximum contaminant level
MCN	micronucleus
µg	microgram
µL	microliter
µM	micromolar
µm	micrometer
mg	milligram
mL	milliliter
mM	millimolar
mm	millimeter
min	minute
MS	mass spectrometry
MTBE	methyl *tert*-butyl ether
MX	3-chloro-4-(dichloromethyl)-5-hydroxy-2-(*5H*)-furanone
NA	not applicable
N-DBP	nitrogen-containing DBP
NS	not statistically significant
PBS	phosphate-buffered saline
%C½ value	the calculated DBP concentration that induced a CHO cell density that was 50% of the negative control
power	the probability that the statistical test will detect a difference if there really is a difference
pH	the logarithm of the reciprocal of hydrogen-ion concentration in gram atoms per liter
p*K*a	the negative logarithm of the equilibrium constant for the dissociation $HA \leftrightarrow H^+ + A^-$
R^2	the fit of the regression analysis
r	Pearsons Product Moment correlation coefficient
RSJ100	*S. typhimurium* strain that expresses GSTT1-1
S9	hepatic 9000 ×*g* supernatant
SAR	structure activity relationship
SCGE	single cell gel electrophoresis
SCE	sister chromatid exchange
S_N2	akalyation potential
SOS	error-prone DNA repair system
.txt	text file
TBAA	tribromoacetic acid
TBAcAm	tribromoacetamide
TBNM	tribromonitromethane
TCAA	trichloroacetic acid
TCAcAm	trichloroacetamide

TCAN	trichloroacetonitrile
TCNM	trichloronitromethane
THM4	the 4 trihalomethanes regulated by the U.S. EPA
TOC	total organic carbon
TOX	total organic halide
$TOX_{chloramines}$	TOX generated after chloramine disinfection
$TOX_{chlorine}$	TOX generated after chlorine disinfection
$TOX_{chlorine\ dioxide}$	TOX generated after chlorine dioxide disinfection
TOX_{ozone}	TOX generated after ozone disinfection
U.S. EPA	United States Environmental Protection Agency

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