MECHANISMS OF MONOHALOGENATED ACETIC ACID INDUCED GENOMIC DNA DAMAGE

BY

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DISSERTATION

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ABSTRACT

Disinfection of drinking water stands among the greatest public health achievements in human history. Killing or inactivation of pathogenic microbes by chemical oxidants such as chlorine, chloramine, or ozone have greatly reduced incidence of waterborne diseases. However, the disinfectant also reacts with organic and inorganic matter in the source water and generates a mixture of toxic disinfection byproducts (DBPs) as an unintended consequence. Since they were first discovered in 1974, over 600 individual DBPs have been detected in disinfected water. Exposure to DBPs is associated with increased risks for developing cancers of the colon, rectum, and bladder, and also for adverse pregnancy outcomes including small for gestational age and congenital malformations. While individual DBPs are teratogenic or carcinogenic, because they are formed at low concentrations during disinfection, it is unlikely that any one DBP can account for these increased risks. While genotoxicity and oxidative stress have been suggested, the mechanisms connecting DBP exposures to adverse health and pregnancy outcomes remain unknown. Investigating mechanisms of toxicity for individual, or classes of DBPs will provide a better understanding of how multiple DBPs interact to generate adverse health and pregnancy outcomes.

Monohalogenated acetic acids (monoHAAs) iodoacetic acid (IAA), bromoacetic acid (BAA), and chloroacetic acid (CAA) are genotoxic and mutagenic with the consistent rank order of toxicity of IAA > BAA > CAA. The comparative toxicity of these compounds was highly correlated with their S_N2 reactivity. The working hypothesis that monoHAAs were directly alkylating DNA was tested and rejected when no damage accumulated in acellular DNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was investigated as a possible molecular target for the monoHAAs; each of the monoHAAs inhibited GAPDH with variable efficacy. The

ability to inhibit GAPDH correlated strongly with multiple toxicological endpoints measured for the monoHAAs. Inhibition of GAPDH was known to disrupt cellular Ca^{2+} homeostasis and generate reactive oxygen species (ROS). The roles Ca^{2+} and ROS played in mode of genotoxic action for the monoHAAs were investigated. Each of the monoHAAs generated biomarkers of oxidative stress, measured both with toxicogenomic analysis and with the production of an antioxidant response element driven reporter gene. The antioxidant butylated hydroxyanisole and the intracellular Ca^{2+} chelator BAPTA-AM reduced genomic DNA damage induced by each of the monoHAAs, supporting the hypothesis that monoHAAs induced genotoxicty by a cascade of events initiated by inhibition of GAPDH.

Disruption of Ca^{2+} homeostasis was a common event in genotoxicity induced by bromacetonitrile (BAN) and bromoacetamide (BAM). Although the BAN and BAM act through depletion of glutathione and BAA acts by inhibiting GAPDH, when BAN or BAM was combined with BAA in a defined component, binary mixture the genotoxicity was additive. These data suggested that the collective action of multiple DBPs acting through different pathways could converge to disrupt Ca^{2+} homeostasis, generate ROS, and cause genomic DNA damage.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Introduction

Disinfection Byproducts and Human Health Risks

Disinfection of drinking water provides an effective means of reducing outbreaks of diseases caused by water-borne pathogens and represents a significant public health achievement of the 20th century [1]. However, when chemicals such as chlorine, chlorine dioxide, chloramines, or ozone are used to kill or inactivate pathogenic microbes they also react with organic matter in the source water. Disinfection byproducts (DBPs), unintended contaminants formed by the reaction of disinfectant, natural organic material (NOM), and inorganic precursors such as iodide (I^-) and bromide (Br^-) in the source water, were first discovered in 1974 when Rook and Bellar independently discovered chloroform and other trihalomethanes (THMs) in chlorinated drinking water [2, 3]. In 1976 a national survey of drinking water facilities found the THMs were ubiquitous in chlorine disinfected water [4]. In the same year data published by the National Cancer Institute reported that chloroform was carcinogenic in laboratory animals [5]. These two discoveries prompted the United States Environmental Protection Agency (U.S. EPA) to pass the first DBP regulation in 1979 with the Interim Trihalomethane Rule, which set a maximum contamination limit (MCL) for total trihalomethanes (TTHMs), including chloroform Cl_3CH, bromodichloromethane $BrCl_2CH$, dibromochloromethane Br_2ClCH, and bromoform Br_3CH, at 100 µg/L [6]. In attempt to further limit the risk associated with DBP exposure, the U.S. EPA extended regulation of DBPs in 1998 with the passage of the Stage I Disinfectants and Disinfection Byproducts (D/DBP) rule. The Stage I D/DBP rule lowered the MCL for TTHMs to 80 µg/L, set MCLs for chlorite and bromate at 1000 µg/L and 10 µg/L respectively, and set an

MCL for five haloacetic acids (HAA5), including bromoacetic acid (BAA), dibromoacetic acid (DBAA), chloroacetic acid (CAA), dichloroacetic acid (DCAA) and trichloroacetic acid (TCAA), at 60 µg/L [7]. The Stage I rule required compliance with all MCLs based on a running annual average of all sampling areas throughout the distribution system. This allowed facilities to deliver water out of compliance to a portion of their distribution system as long as the average across the total distribution system remained in compliance. To address this problem the U.S. EPA passed the Stage II D/DBP rule, which maintained the MCLs set in the Stage I rule, but required each monitoring station to remain in compliance over a one year average [8].

Since the discovery of the first DBPs in 1974 [2, 3] over 600 individual compounds have been identified in disinfected drinking water [9]. The complexity of the organic materials along with many other variables including, temperature, pH, concentrations of Br^- and I^- in the source waters, type and concentration of disinfectant used, and contact time with the disinfectant lead to complex mixtures of DBPs that can vary significantly among distribution systems, and also within a single system [10, 11]. These complex mixtures vary both in the individual chemicals generated as well as the concentrations in which each chemical is formed [12, 13]. A review of the occurrence, toxic properties, and carcinogenicity of individual DBPs is available [14].

Concerns over the toxicity and carcinogenicity of individual DBPs prompted several epidemiological studies in an attempt to evaluate the health effects of exposure to the complex mixture of chemicals formed by water disinfection. Many of these studies found that exposure to disinfected water was associated with increased risk of colon cancer [15-17], bladder cancer [18-25], and adverse pregnancy outcomes including small for gestational age and premature delivery [26-28]. The U.S. EPA estimated that the population risk attributed to chlorinated water accounted for 2% - 17% bladder cancer cases in the United States (US) [29]. However, based on

animal carcinogenicity data, none of the regulated DBPs can account for the increased bladder cancer risk seen in the epidemiologic studies [30]. Thus, a gap in the knowledge connecting DBP exposure to adverse health and pregnancy risk persists.

While disinfection of drinking water has doubtlessly increased life expectancy and quality of life by preventing acute disease caused by pathogens in untreated water, the use of disinfectants created a situation in which virtually all of the population in the developed world is chronically exposed to complex mixtures of chemicals with known adverse health outcomes. While the induction of genotoxicity and oxidative stress among others were proposed, the mechanisms linking toxicity of individual or total DBP exposure to increased risks of cancer or adverse pregnancy outcomes are not defined [31]. Additional research is needed to identify the specific causes of these adverse effects. Understanding the mechanisms by which individual DBPs induce their toxic effects is essential to gain insight into additive or synergistic effects and might more accurately account for measured adverse health effects associated with disinfected water. Identification of cellular mechanisms of DBP-mediated toxicity may also help to identify populations susceptible to the effects of exposure due to genetic polymorphisms of specific genes (e.g. those involved in metabolic function, DNA repair, and/or responses to oxidative stress).

Haloacetic Acids

One of the most abundant classes of DBP found in disinfected water is the haloacetic acids (HAAs) [10, 12]. HAAs were the second most prevalent class of DBP, accounting for 15.4 % of the total organic halide (TOX) fraction extracted from chlorinated drinking water, and the most prevalent class in chloraminated water, accounting for 15.4% of the TOX fraction [10].

The di- and tri-halogenated acetic acids with multiple chlorine, or mixed chlorine and bromine substituted species are generally the most abundant, however, in source waters with high Br^- and I^-, brominated DBPs can be the dominant species [13]. In a survey of 12 water distribution plants in the US described as having high precursor loads, the sum of 9 HAAs (HAA9) (including CAA, DCAA, TCAA, BAA, DBAA, tribromo (TBAA), bromochloro (BCAA), bromodichloro (BDCAA), and dichlorobromoacetic (DCBAA) acids) ranged from 5 µg/L to 130 µg/L with a median of 34 µg/L [13]. A survey of drinking water in 11 provinces in Spain found a median HAA9 concentration of 29.0 µg/L (ranging from 18.9 µg/L to 53.1 µg/L); in these samples CAA and BAA were below detection limits of 2 µg/L and 1 µg/L, respectively and iodoacetic acid (IAA) was not measured [32]. The sum of 6 HAAs including TCAA, BCAA, BCAA, DBAA, DBCAA, BAA were measured in 3 regions in England, the mean for each system was 35.1 µg/L, 52.1 µg/L, and 94.6 µg/L with a maximum measurement of 244 µg/L; mean BAA concentrations were 2.6 µg/L, 4.0 µg/L, and 1.7 µg/L with a maximum of 17 µg/L [33]. Neither CAA nor IAA was measured in this study. In Pearl River Delta cities Guangzhou, Foshan, and Zhuhai in Gangduong Province China HAA9 ranged from 0.3 – 81.3 µg/L with DCAA and TCAA being the most abundant species; CAA was below the detection limit (2 µg/L) in all waters sampled [34].

IAA was first discovered in drinking water in a nationwide occurrence study in the US [13], however only limited data are available for its occurrence. One recent survey of DBP occurrence in Chinese water systems included measurements for IAA. Wie et al investigated 13 drinking water plants in Shanghai China, all of which used chloramines as the primary disinfectant, whose source waters were the Yangtze or Huangpu rivers [35]. In these waster systems HAA9 ranged from 3.31 µg/L to 48.55 µg/L; IAA was detected in all systems ranging

from 0.04 µg/L to 1.66 µg/L [35]. The maximum IAA concentration (1.66 µg/L) occurred in chloraminated water sourced from the Huangpu River, which had high I^- concentrations (up to 18 µg/L) relative to the Yangtze [35].

Each of the five regulated HAAs (CAA, DCAA, TCAA, BAA, and DBAA) was mugagenic in *Salmonella typumirium* [36], genotoxic in Chinese hamster ovary cells [37], and teratogenic in whole mouse embryo cultures [38]. DCAA [39-41], TCAA [40], and DBAA [42] were carcinogenic in rodent bioassays, but CAA was not [43, 44]. No carcinogenicity data are presently available for BAA despite its status as a regulated compound.

In vitro studies showed that BAA was consistently more toxic than DBAA, and CAA was more toxic than DCAA and TCAA, indicating that the monoHAAs are more toxic then their di or tri halogenated analogues [36-38]. Comparative studies also showed a consistent trend among the monoHAAs where IAA is the most and CAA is the least toxic, indicating that the halogen substituent plays an important role in toxicity [36, 38, 45, 46].

The focus of this research is on the HAAs with a single halogen substituent, including IAA, BAA, and CAA which will be referred to collectively as monoHAAs. The structures of the monoHAAs are displayed in Figure 1.1. While di- and tri-substituted haloacetic acids were the largest component of the total HAA fraction [32, 34, 47, 48], the monoHAAs were the most genotoxic [49] and teratogenic [38, 45] when comparative analyses were performed. Additionally IAA and BAA were the most mutagenic of the HAAs. By focusing on the monoHAAs, the mechanism(s) of toxicity can be evaluated in a series of 3 compounds with a single variable in the chemical structures and the role the halogen substituent plays in toxicity can be assessed. The toxicity of the monoHAAs reported in the literature is reviewed below.

Because DBP exposures are associated with cancer and adverse pregnancy outcomes, the toxic endpoints included in the review were limited to those relevant to these epidemiologic endpoints.

Genotoxicity, Mutagenicity, and Carcinogenicity of the monoHAAs

Mutagenicity

Analyses of mutagenicity associated with CAA using *S. typhumirium* generated mixed, but mostly negative results. BAA and IAA, while less studied, were consistently positive as direct-acting mutagens *S. typhumirium*. Each of the monoHAAs was mutagenic in mammalian cells, although data are limited. Table 1.1 summarizes the results of published mutagenicity assays involving the monoHAAs.

Salmonella Typhumirium Mutagenicity

CAA did not significantly increase mutation frequencies in *S. typhumirium* tester strain TA 1535 [50]. Additionally, CAA was negative in the presence and absence of exogenous metabolic (S9) activation in *S. typhumirium* strains TA100, TA 98, TA1535, and TA 1537 [51]. Conversely, using a modified suspension assay Kargalioglu et al reported that CAA increased mutations in *Salmonella* TA 100 with and without S9 (+ S9, 12-25 mM; -S9, 14-24 mM), and also in TA98 (20-28 mM) without S9 activation [36]. This study also reported that BAA was mutagenic in TA 98 with and without S9 (+ S9, .472 mM; -S9, 0.272 mM) and TA 100 with and without S9 activation (0.222 mM for both) [36]. Giller et al reported that BAA, but not CAA was mutagenic in TA 100 +S9 [52]. Iodoacetic acid was mutagenic in *Salmonella* TA 100 (>70 µM) [46]; the mutagenicity induced by IAA was decreased in the presence of antioxidants [53]. The

descending rank order in mutagenic potency among the 3 monoHAAs in TA 100 was IAA > BAA >> CAA (Figure 1.2) [46].

Mammalian Cell Mutagenicity

CAA with S9 was weakly mutagenic in the mouse lymphoma (L5178Y) thymidine kinase (Tk $^{+/-}$) mutation assays [54, 55]. However, at the mutagenic concentration (400 µg/mL), an acidic pH shift that could have influenced the results was noted [55]. Zhang et al, using the hypoxanthine–guanine phosphoribosyltransferase (HGPRT) gene mutation assay, showed that each of the monoHAAs were mutagenic in CHO K1 cells, with the rank order of mutagenicity being IAA > BAA > CAA [56].

Genotoxicity/DNA Damage

DNA damage can be measured through reporter based assays in which a gene for a reporter protein is attached to DNA damage response element (a cis regulatory element in DNA) that responds to damage by increasing transcription and translation of the reporter, thereby producing a measurable signal in response to DNA damage. DNA damage can also be measured directly as strand breaks, sister chromatid exchange (SCE), chromosomal aberrations, or micronuclei. Only limited data are available for reporter based DNA damage assays with both CAA and BAA being negative. Direct measures of DNA damage generated by the monoHAAs are more abundant. Measures of clastogenicity were mixed for each of the monoHAAs. Each of the monoHAAs induced genomic DNA damage, measured by the alkaline single cell gel electrophoresis (SCGE) assay in multiple cell lines, suggesting that these compounds are

genotoxic to mammalian cells. Table 1.2 summarizes the published genotoxicity data for the monoHAAs.

Bacteria

CAA in the presence or absence of S9 did not activate DNA repair in the S. typhumirium TA1535/pSK1002 containing the umuC'-'lacZ reporter gene [57]. Neither CAA nor BAA, at concentrations up to 3000 µg/mL, was positive using the SOS chromotest in the tester strain Escherichia coli PQ 37 [52].

Mammalian Cells

BAA induced DNA single strand breaks measured with the alkaline elution assay in the murine neuroblastoma cell line L-1210 [58]. CAA did not induce DNA strand breaks in liver or splenocytes, or epithelial cells isolated from the stomachs or duodenums of Male Fischer 344 rats and male B6C3Fl mice, or the human lymphoblastic cell line CCRF-CEM when measured with the DNA alkaline unwinding assay [59]. In the same study CAA (5 mM and 10 mM) induced strand breaks in primary hepatocytes isolated from the F334 rats, however, the authors suggested this could have been caused by acute cytotoxicity as cell viability was significantly decreased under the same conditions [59]. CAA did not induce chromosomal aberrations or sister chromatid exchanges in Chinese hamster lung fibroblasts [60]. In another study CAA without (but not with) S9 induced SCE in Chinese hamster ovary (CHO) cells but did not generate chromosomal aberrations [61]. CAA, BAA, and IAA induced chromatid, but not chromosomal type aberrations in primary human lymphocytes [62]. Neither CAA nor BAA generated micronuclei in newt (*Pleurodeles wall*) larvae [52]. CAA, BAA, and IAA also did not induce micronuclei in the human lymphoblast (TK 6) cell line [63].The sodium salt of IAA (sodium

iodoacetate) did not produce SCE or chromosomal aberrations in Chinese hamster lung fibroblast (V79) cells [64]. In another study, iodoacetate generated chromosomal aberrations in CHO cells with and without S9 activation, and also in TK6 cells; however, the authors concluded that the effects were likely associated with cytotoxicity rather than direct interaction with DNA [65].

CAA, BAA, and IAA induced genomic DNA damage measured with the alkaline single cell gel electrophoresis assay (SCGE) in CHO cells (Figure 1.3) [37, 46], in nontransformed human small intestine epithelial cells (line FHs 74 Int) (Figure 1.4) [66], and in primary human lymphocytes [62]. IAA and BAA, but not CAA generated DNA damage, measured with SCGE, in and in the human liver carcinoma cell line HEPG2 [67]. A study by Wei et al found that IAA generated genomic DNA damage in NIH 3T3 cells measured with both γH2AX staining and SCGE, but did not generate micronuclei [68]. Iodoacetate was genotoxic to CHO K5 cells, but did not induce damage to DNA isolated from CHO K5 except at the highest concentration tested (10 µg/mL); the authors suggested that the small but significant increase in DNA migration at this concentration was "fortuitous and not compound related" [69]. This finding was supported by an additional study which reported that none of the monoHAAs generated DNA damage in isolated CHO DNA, including IAA at concentrations up to 500 µM [70]. Studies that have quantitatively compared the genotoxicity of the monoHAAs have consistently found the genotoxic potency rank order to be IAA> BAA > CAA (Figures 1.3 and 1.4) [46, 62, 66, 71]. DNA damage induced by the monoHAAs in CHO cells was repaired under liquid holding conditions, suggesting that the DNA lesions were not lethal, and could be repaired [71].

Carcinogenicity

Carcinogenicity data is limited for the monoHAAs. Only CAA has been tested in a 2 year rodent carcinogenicity assay. CAA, in deionized water, at concentrations up to 30 mg/kg/day administered to F334/N rats, or at concentrations up to 100 mg/kg/day administered to B63F1 mice by gavage generated no carcinogenic activity [44]. An additional study in which CAA at concentrations up to 59.9 mg/kg/day was administered to F334/N rats in drinking water also found no carcinogenic activity [43].

IAA, when applied to the skin, promoted tumors in "S" strain albino mice pretreated with a single application of 9,10-dimethyl-1,2 benzanthracene [72]. IAA induced malignancy in NIH3T3 cells with anchorage independent growth and agglutination with concavalin A [68]. The NIH3T3 cells transformed by IAA exposure formed fibrosarcomas after injection into Balb/c nude mice [68]. This study showed that IAA generated malignancy *in vitro,* but further evaluation in an *in vivo* cancer bioassay is needed to evaluate IAA's status as a human carcinogen.

Reproductive Toxicity and Teratogenicity

In Vivo

While DBP exposure is weakly associated with adverse pregnancy outcomes, the reproductive and or teratogenic effects of the monoHAAs have only scarcely been evaluated. In one study, pregnant Sprague Dawley dams were given CAA (1570 ppm) in drinking water (average dose reported as 33 mg/rat/d) from gestational day 1 – 22, cardiac defects as well as changes in live/dead pups, fetal weight, placental weight, crown rump length, and external morphology were examined with no significant effects observed [73]. Linder et al studied the

acute spermatogenic effects of BAA in male Sprague Dawley rats dosed by gavage either as a single dose of 100 mg/kg or 14 doses of 25mg/kg/day; no significant effects were observed [74]. Pregnant CFW mice given intramuscular injections of 0.5 mg IAA in distilled water at gestational day 11 or 13, or days 11-13 or 12-14 significantly increased the incidence of cleft palate in the pups; administration of sodium succinate (2 mg) but not sodium pyruvate (2 mg) reduced the effect of the IAA injection on days 11-13 [75]. Fasting the pregnant dams on gestational day 13, days 11-12, or days 11-13 also induced cleft palates in the pups; addition of IAA to the fasting did not increase cleft palate incidences, the authors of this study concluded that the fasting and IAA were acting at different steps of the same metabolic pathway [75]. Because succinate which effectively increased energy production during fasting , but not pyruvate, reduced the effect of treatment the authors suggested that IAA and fasting were both inducing their teratogenic effect by disrupting the metabolism of glucose "from the beginning of glycolysis to the dehydrogenation of succinate" [75].

In Vitro

In a series of *in vitro* whole mouse embryo studies each of the monoHAAs generated neural tube defects (NTD) [38, 45]. The lowest concentrations that significantly increased the incidences of NTD were 2.5 μM IAA [45], 6 μM BAA and 175 μM CAA [38]. In addition to NTD, BAA also induced rotational defects, pharyngeal arch defects and heart defects at tested concentrations ≥ 6 μM, somite dysmorphology at 8 μM, and eye deformations at 10 μM; CAA induced pharyngeal arch and heart defects at 250 μM [38].

Epidemiology

Because the HAAs have only been regulated in the US since 1998, the lack of consistent monitoring of HAA levels in drinking water prior to this time prevents accurate exposure assessment in cancer epidemiology. However, multiple human epidemiologic studies have evaluated the relationship between HAA exposure and adverse pregnancy outcomes [76-81], neural tube defects [82], or semen quality [83]. Among these only the study by Porter et al attempted to evaluate the relationship between single monoHAAs (CAA, and BAA) and any pregnancy outcome [78]. The authors reported that BAA exposures from the 3rd and 4th quintiles during 2nd trimester of pregnancy resulted in significantly increased risk of intrauterine growth retardation (IGR) OR 1.3 (1.02- 1.65), and 1.3 (1.02-1.66), respectively; however there was no increased risk associated with these or any other monoHAA exposure level when evaluated over the entire length of the pregnancy [78]. While there was no measurement of any of the individual monoHAAs, the study by Klotz et al [82] was of particular interest because the endpoint they evaluated, neural tube defects (NTD), had been previously associated with monoHAA exposures *in vitro* [38, 45]. Although an increase in NTD was associated with TTHMs was discovered when the highest (> 40 ppb) vs. the lowest (< 5 ppb) tertiles were compared, this study found no association with total HAA exposure during the first trimester of pregnancy and NTD [82]. Among the studies that evaluated risk associated with total HAA exposure, only one reported increased risk for any adverse pregnancy outcome (small for gestational age) which was associated with the sum of the 5 HAAs (CAA, BAA, dichloro-, trichloro- and dibromoacetic acid) (HAA5) over 60 µg/L vs < 60 µg/L, OR 1.4 (1.1 – 1.9) [81].

Mechanism(s) of Toxicity

Several comparative studies that evaluated the toxicity of the monoHAAs found that these compounds were mutagenic in *S. typhimurium* [36, 46], cytotoxic and mutagenic in Chinese hamster ovary (CHO) K1 cells [56], cytotoxic and genotoxic in CHO AS52 cells [37, 46, 71] genotoxic in human FHs cells [66], genotoxic in human lymphocytes [62], cytotoxic but not clastogenic in human TK6 cells [63], and teratogenic in *in vitro* whole mouse embryos [38, 45]. In each case the rank order of toxicity was IAA > BAA > CAA.

The monoHAAs are electrophilic alkylating agents; the electron withdrawing effects of the carboxylic acid functional group as well as the halogen substituent leave the carbons electron deficient and open for nucleophilic attack. The halogen substituent, through bond dissociation energy and other factors, determines the relative bimolecular nucleophilic substitution (S_N2) reactivity of the compound, and the relative S_N2 reactivity is predictive of their toxicity in mutagenic, genotoxic, cytotoxic, and teratogenic endpoints [46]. Essentially, the iodine is a better leaving group than chlorine in the S_N2 reaction, which enhances the ability of the compound to alkylate a biological nucleophile and cause toxicity. Which biological nucleophile(s) is being alkylated is the focus of the remainder of the research reported in this dissertation.

Neurotoxicity of IAA

The neurotoxic effect of IAA has been the most rigorously studied toxicological mechanism among the three monoHAAs. IAA has long been known to inhibit the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [84]. An S_N2 reaction between the electron rich thiolate sulfur and IAA's electrophilic α-carbon results in a carboxymethylation

of the catalytic cysteine residue and irreversibly inhibits the enzyme's activity [85]. The ability to inhibit glycolysis has made IAA a useful model to study the effects of ischemia/reperfusion injury in neurotoxicology. Studies using this model of chemical ischemia show a drop in cellular ATP level [86-88], an increase in cytosolic free calcium ions (Ca^{2+}) [86-88], release of free arachidonic acid (AA) [88, 89], generation of ROS [86, 88, 90], and cell death [86, 87, 91] subsequent to IAA exposure. These studies have suggested that increases in cellular calcium levels, phospholipid degradation, and ROS formation are all critical events in the toxic cascade, as antioxidants [36-41], phospholipase inhibitors [40, 42], and calcium free medium or calcium chelators can reduce or delay the toxic response [38]. It is important to note that the rise in Ca^{2+} generated from 100 μM IAA was from an intracellular source rather than through N-methyl-D-aspartate (NMDA) receptor mediated Ca^{2+} influx [86, 87].

Free Ca^{2+} is present at very low concentrations in the cytosol (~100 nM), and a large gradient, roughly 20,000 fold, is maintained across the cellular membrane; additionally Ca^{2+} is stored in the mitochondria and endoplasmic (ER) or sarcoplasmic reticulum (SR,) again with a large gradient across the organellar membranes [92-94]. Maintenance of these gradients requires a substantial amount of energy, which, in the cell, is generally provided by hydrolysis of the high energy phosphoanhydride bonds in adenosine triphosphate (ATP) [93]. Glycolytic enzymes were found coordinated on the SR membrane and provided ATP derived specifically from glycolysis in the proximity of the ER/SR calcium ATPase (SERCA), a pump responsible for maintaining Ca^{2+} homeostasis within the ER/SR [95]. SERCA activity was significantly reduced after exposing cardiac myocytes or isolated SR preparations to IAA [95]. In this study, addition of exogenous ATP or phosphoenolpyruvate, a glycolytic intermediate (downstream of GAPDH) restored SERCA activity, suggesting it was not directly inhibited by IAA [95]. These findings

were supported in a neuronal cell model when Ca^{2+} leaked from the ER of hippocampal neurons after exposure to glycolytic inhibitors including IAA [96]. The Ca^{2+} specific intracellular chelator, 1,2- bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM), reduced the production of ROS and neuronal cell death associated with IAA exposure, suggesting a relationship between a rise in intracellular Ca^{2+} and oxidative stress [86, 87]. Taken together, these findings, at least in theory, provided a functional connection between inhibition of glycolysis, Ca^{2+} leakage from ER, and the generation of ROS and suggested that toxicity associated with IAA might be due to inhibition of SERCA. In support of this hypothesis, thapsigargin, a specific irreversible inhibitor of SERCA, resulted in the release of free AA [97, 98], and induced the formation of ROS [98], events also present in the toxic cascade initiated by IAA in neurons. Thapsigargin also did not potentiate the neurotoxic effect of IAA, again suggesting that the two chemicals were working at different steps in the same pathway [87].

Mutagenicity/Genotoxicity and Teratogenicity

When BAA generated strand breaks in mouse lymphoma cells, the authors hypothesized that direct alkylation of DNA could be responsible [58]. These data agreed with the high correlation of DNA damage induced by the monoHAAs with their respective alkylating potentials [46]. In *Salmonella* and other biosystems, where the monoHAAs were mutagenic without metabolic activation (by definition direct-acting), seemed to agree that a direct alkylation of DNA might be occurring, or at least the monoHAA, rather than some metabolic byproduct, was responsible for toxicity. The authors suggested cytotoxicity, rather than direct alkylation, was responsible for the damaged DNA in study in which iodoacetate induced chromosomal

aberrations, [65]. Kiffe et al designed a set of experiments to determine if iodoacetate was directly alkylating DNA using the standard alkaline SCGE assay and a modified version of SCGE in which the cells were lysed before exposure leaving only the DNA to interact with the toxicant [69]. CHO K5 cells exposed to iodoacetate accumulated significant damage in their DNA; however, the acellular DNA did not [69]. These data suggested that IAA did not directly alkylate DNA and that some cellular process was required for iodoacetate to generate genotoxicity. However, the high correlation between genomic DNA damage and alkylating potential suggested that alkylation of some cellular component was involved in the origin of the genotoxicity [46].

Reactive oxygen species (ROS), especially hydroxyl radical (OH·), can react with DNA resulting in lesions such as 8-hydroxydeoxyguanosine (8-OH-dG) making the generation of ROS a viable source of mutagenicity and genotoxicity [99, 100]. Malondialdeyde (MDA), a product of lipid peroxidation and a byproduct of ROS production, was also mutagenic [101]. Organic extracts from chlorinated water increased MDA concentrations and reduced levels of glutathione (GSH), a major component of the antioxidant defense system, in human liver cells; these observations suggested that these extracts were generating oxidative stress [102, 103]. In these studies both MDA and GSH measurements were highly correlated with DNA damage [102, 103]. In another study, chlorination of drinking water increased oxidative stress measured by activation of a reporter gene attached to an antioxidant response element (ARE) [104]. Cemeli et al, found that the antioxidant enzyme catalase, as well as the radical scavenger butylated hydroxyanisol, reduced the mutagenicity and genotoxicity of IAA, suggesting that ROS might be involved DNA damage and mutagenicity associated with this toxicant [53]. Two toxicogenomic analyses also suggested a role for ROS in monoHAA induced toxicity, when it was discovered

that each of the monoHAAs altered transcription of genes associated with DNA double strand breaks [66, 105]. Alkylated bases generally do not result in double strand breaks prior to replication [106]. ROS, however, are known to generate DNA double strand breaks, as highly reactive hydroxyl radicals are formed from H_2O_2 via the Fenton reaction catalyzed by metal ions present in DNA induce strand breaks [107]. The localization effect of the metal ions and the reactivity of the hydroxyl radical create hotspots where multiple lesions are likely to occur in close proximity of the metal ion, thus two single events have an increased probability of generating a double strand breaks [107].

ROS can also negatively impact fetal development by altering critical signaling pathways through oxidation [59]. Oxidative stress during organogenesis modifies redox-regulated transcription factors and consequently modifies gene expression during development [108]. Biomarkers of oxidative stress such as 8-OH-dG and MDA found in the urine of pregnant women were associated with decreased birth weight and premature delivery [109] as well as small for gestational age [110], outcomes which have also been associated with DBP exposure. The teratogenic effect of IAA was mirrored by exposure to 2-deoxyglucose (2DG) a competitive inhibitor of glycolysis and also by fasting pregnant dams , indicating that disrupting the glycolytic pathway has a detrimental effect on fetal development [45, 75].

IAA induces a toxic cascade in neurons that is initiated by the inhibition of GAPDH. Subsequent to GAPDH inhibition ATP levels drop leading to an inability to maintain Ca^{2+} gradients, which in turn leads to the formation of ROS, phospholipid degradation, and ends in neuronal death [86-89]. Disruption of glycolysis also seems to be an important factor in monoHAA induced teratogenicity [38, 45]. It is not clear that all cell types are equally susceptible to GAPDH inhibition. For instance, Kumagai et al showed that CHO K1 cells were

less susceptible than cancer cell lines, which are highly dependent on glycolysis, to toxicity induced by koningic acid, a potent inhibitor of GAPDH [111]. We previously demonstrated that the HAAs, including IAA, induce toxicity in CHO AS52 cells [37, 46]. However, it remains to be determined if the same toxic cascade observed in IAA induced neurotoxicity is involved in the genotoxic/mutagenic responses induced by each of the monoHAAs in the mammalian and bacterial cell models used in our laboratory. The research described herein attempts to address these knowledge gaps and to explore how the monoHAAs generate their toxic responses, and more importantly how these compounds contribute to known adverse health effects associated with disinfected water.

Rationale for Research

Exposure to DBPs is an unintended but necessary consequence of drinking water disinfection. While greatly reducing the acute risk of exposure to water-borne pathogens [1], epidemiologic studies suggest that disinfecting water increases the risk of bladder [18-25] and colon cancers [15-17] and adverse pregnancy outcomes [26-28]. Although genotoxicity and oxidative stress were proposed [31], there is no clear mechanism leading from exposure to these adverse health and pregnancy outcomes. Additionally the modes of action for the toxicity of many individual DBPs have not been defined. None of the DBPs that were carcinogenic in rodent assays generated bladder cancer and the concentrations that generated neoplasms were much higher than those that would occur through drinking water [30]. Additional research into the toxic mechanisms of individual DBPs would reduce the current knowledge gap between DBP exposure and the associated health outcomes by defining possible areas of additivity among multiple components of the total DBP exposure. The HAAs were a significant fraction of the

total DBP pool [13]. Within the larger class of HAAs, the monoHAAs were consistently the most toxic [37, 38, 45, 46]. These compounds are electrophilic, and their relative S_N2 reactivity was predictive of their toxicity, that is, in the case of both reactivity and toxicity the rank order is IAA > BAA > CAA [46]. The biological nucleophile(s) with which they interact are not clearly defined. Some studies suggested that IAA induced neurotoxicity by specifically inhibiting GAPDH, which then leads to an increase in cytosolic Ca^{2+}, free AA, and the generation of ROS [86, 88]. Additionally, the mutagenicity and genotoxicity associated with IAA seems to involve ROS [33]. The hypothesis that each of the monoHAAs generates genotoxicity by inhibiting GAPDH was investigated in the research reported in this dissertation. Additionally the involvement of Ca^{2+} and ROS in the manifestation of DNA damage was evaluated.

Research Objectives

In an effort to test the hypothesis that the monoHAAs induce genomic DNA damage by inhibiting GAPDH, which then initiates an increase of free Ca^{2+} in the cytosol triggering the formation of ROS the following experiments were conducted:

- Analyze the induction of genomic DNA damage on acellular DNA for each of the monoHAAs. (Chapter 2)
- Measure GAPDH enzyme kinetics in the presence and absence of each monoHAA. (Chapter 2)
- Investigate the cellular response to monoHAA exposure using the ARE-bla HepG2 Reporter Gene Assay. (Chapter 3)

- Investigate cellular responses to monoHAA exposure with toxicogenomic analysis using a Human Oxidative Stress and Antioxidant Defense pathway specific PCR array. (Chapter 3)
- Evaluate genotoxicity in CHO cells treated with individual monoHAAs in the presence and absence of the antioxidant butylated hydroxyanisole (BHA). (Chapter 4)
- Evaluate genotoxicity in CHO cells treated with individual monoHAAs in the presence and absence of the intracellular Ca^{2+} chelator BAPTA-AM. (Chapter 4)

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Tables and Figures

Table 1.1. Summary of published monoHAA mutagenicity assay results.				
Chemical	Biosystem	Genetic Endpoint	Lowest Genotoxic Concentration	Ref
CAA	Salmonella TA 1535 (-S9)	His Reversion	Negative	50
	Salmonella TA 98, TA 100, TA 1535, TA 1537	His Reversion	Negative in all strains ± S9	51
	Samonella TA 98, TA 100	His Reversion	TA 98 -S9, 20 mM; +S9, Negative TA 100 -S9, 14 mM; + S9, 12 mM	36
	Salmonella TA 100	His Reversion	TA 100 -S9, negative; +S9, 20 µg/mL	52
	L5178Y/$Tk^{+/-}$	$Tk^{+/-} \rightarrow TK^{-/-}$	positive +S9	54
	L5178Y/$Tk^{+/-}$	$Tk^{+/-} \rightarrow TK^{-/-}$	400 µg/mL	55
	CHO K1	HGPRT	1 mM	56
BAA	Salmonella TA 100	His Reversion	TA 100 -S9, negative; + S9, 20 µg/mL	52
	CHO K1	HGPRT	800 µM	56
	Salmonella TA 100	His Reversion	TA 98 -S9, 0.272 mM; + S9, 0.472 mM TA 100 -S9, 0.222 mM; + S9, 0.222 mM	36
IAA	Salmonella TA 100	his reversion	TA 100 -S9, 70 µM	46
	CHO K1	HGPRT	30 µM	56

Table 1.2. Summary of published monoHAA genotoxicity assay results.				
Chemical	Biosystem	Genetic Endpoint	Lowest Genotoxic Concentration or Genotoxic Potency (GP)	Ref
CAA	S. typhimirium TA1535/pSK1002	umuC'-'LacZ	Negative ± S9	57
	Escherichia coli PQ 37	SOS chromotest	Negative ± S9	52
	CCRF-CEM	DNA Strand Breaks (DAUA[1])	Negative	59
	B6C3F1 mice (liver, splenocytes, stomach/duodenal epithelial cells)	DNA Strand Breaks (DAUA)	Negative	59
	F334 rats (liver)	DNA Strand Breaks (DAUA)	Negative	59
	F334 rat primary hepatocytes	DNA Strand Breaks (DAUA)	5 mM	59
	Chinese hamster lung fibroblasts	Chromosome aberrations SCE[2]	Negative Negative	60
	CHO	Chromosome aberrations	Negative	61
	CHO	SCE	-S9, 160 µg/mL; +S9 Negative	61
	Primary human lymphocytes	Chromatid aberrations Chromosome aberrations	180 µM Negative	62
	Pleurodeles walll larvae (newt)	Micronuclei	Negative	52
	TK6	Micronuclei	Negative	63
	CHO AS52	SCGE	0.411 mM (GP)	37
	FHs 74 Int	SCGE	1.04 mM (20% Tail DNA)	66
	Primary human lymphocytes	SCGE	805.8 µM (GP)	62
	HEPG2	SCGE	Negative	67
	CHO AS52 Acellular DNA	SCGE	Negative	70
BAA	*Escherichia coli* PQ 37	SOS chromotest	Negative ± S9	52
	L-1210	DNA Strand Breaks (DAUA)	100 µM	58
	Human Lymphocytes	Chromatid aberrations Chromosome aberrations	4 µM Negative	62
	Pleurodeles walll larvae (newt)	Micronuclei	Negative	52
	TK6	Micronuclei	Negative	63
	CHO AS52	SCGE	0.017 mM (GP)	37
	FHs 74 Int	SCGE	23.8 uM (20% Tail DNA)	66
	Human Lymphocytes	SCGE	12.07 µM (GP)	62
	CHO AS52 DNA	SCGE	Negative	70
	HEPG2	SCGE	0.1 µM	67

Table 1.2. (cont.)				
	CHO AS52 Acellular DNA	SCGE	Negative	70
IAA	TK6 (toxicity)	Chromosome aberrations	20 µM	65
	CHO (clone WBL)	Chromosome aberrations	40 µM	65
	TK6	SCE	Negative	63
	Human Lymphocytes	Chromatid aberrations Chromosome aberrations	45 µM Negative	62
	Chinese hamster lung fibroblast (V79) cells [3]	SCE Chromosome aberrations	Negative Negative	64
	NIH3T3	Micronuclei	Negative	68
	CHO AS52	SCGE	8.7 µM (GP)	46
	FHs 74 Int	SCGE	5.9 uM (20% Tail DNA)	66
	Human Lymphocytes	SCGE	10.43 µM (GP)	62
	HEPG2	SCGE	0.01 µM	67
	NIH3T3	SCGE γ-H2AX	3 µM 2µM	68
	CHO K5 [3]	SCGE	0.625, 2.5, 5, and 10 µg/mL	69
	CHO K5 Acellular DNA [3]	SCGE	10 µg/mL (not compound related)	69
	CHO AS52 Acellular DNA	SCGE	Negative	70

[1] DNA alkaline unwinding assay (DAUA); [2] Sister chromatid exchange (SCE); 3Sodium iodoacetate used in place of IAA.

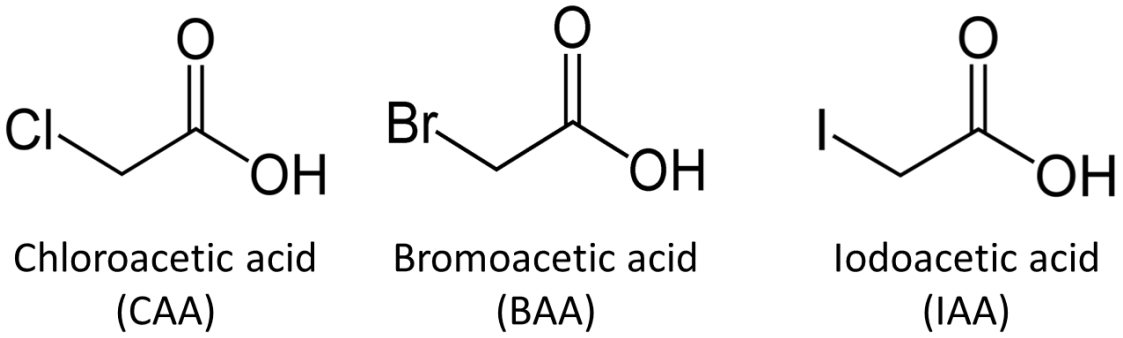

Figure 1.1. Structures of the monoHAAs.

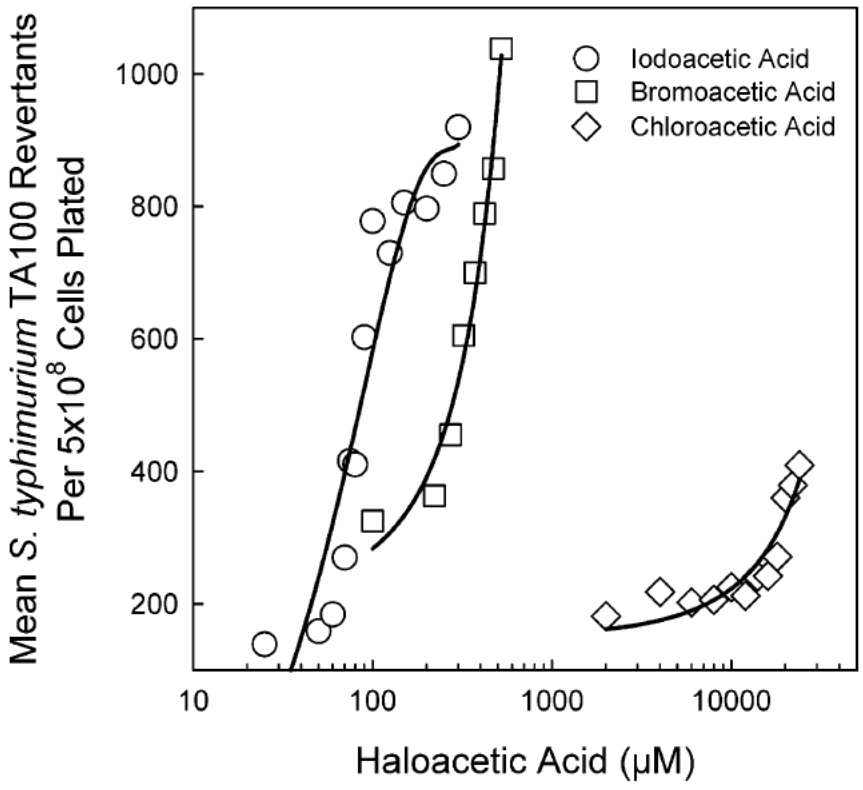

Figure 1.2. Concentration-response curves illustrating the relative mutagenicity of iodoacetic, bromoacetic, and chloroacetic acids in *S. typhimurium* strain TA100 [46].

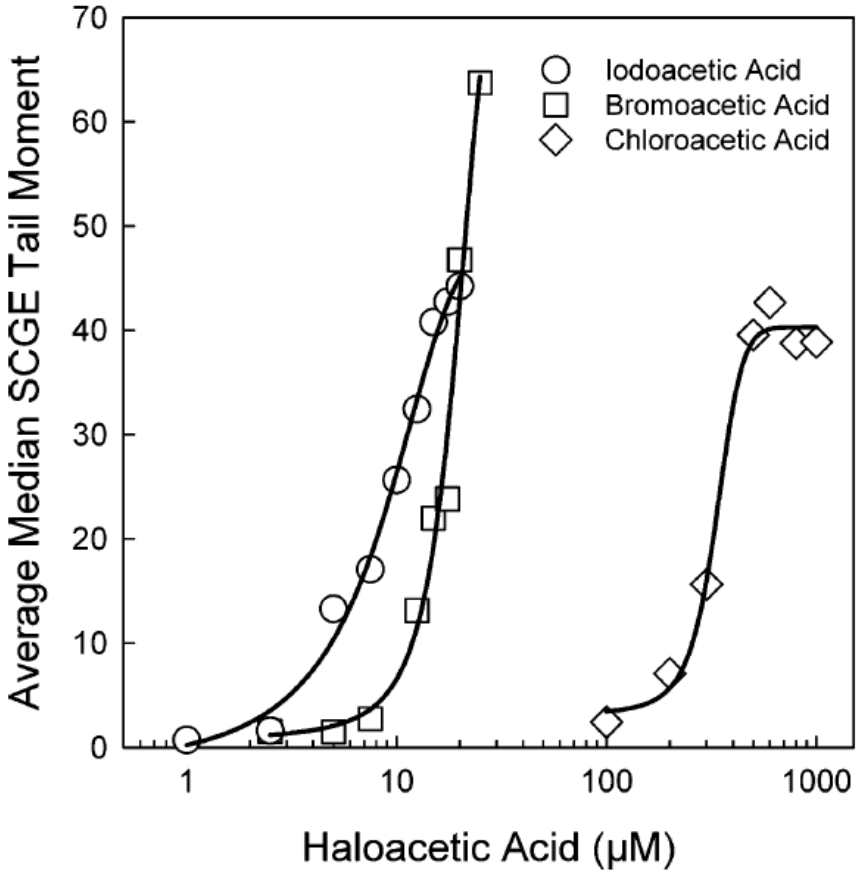

Figure 1.3. Concentration-response curves illustrating the relative levels of genomic DNA damage induced by iodoacetic, bromoacetic, and chloroacetic acids [46].

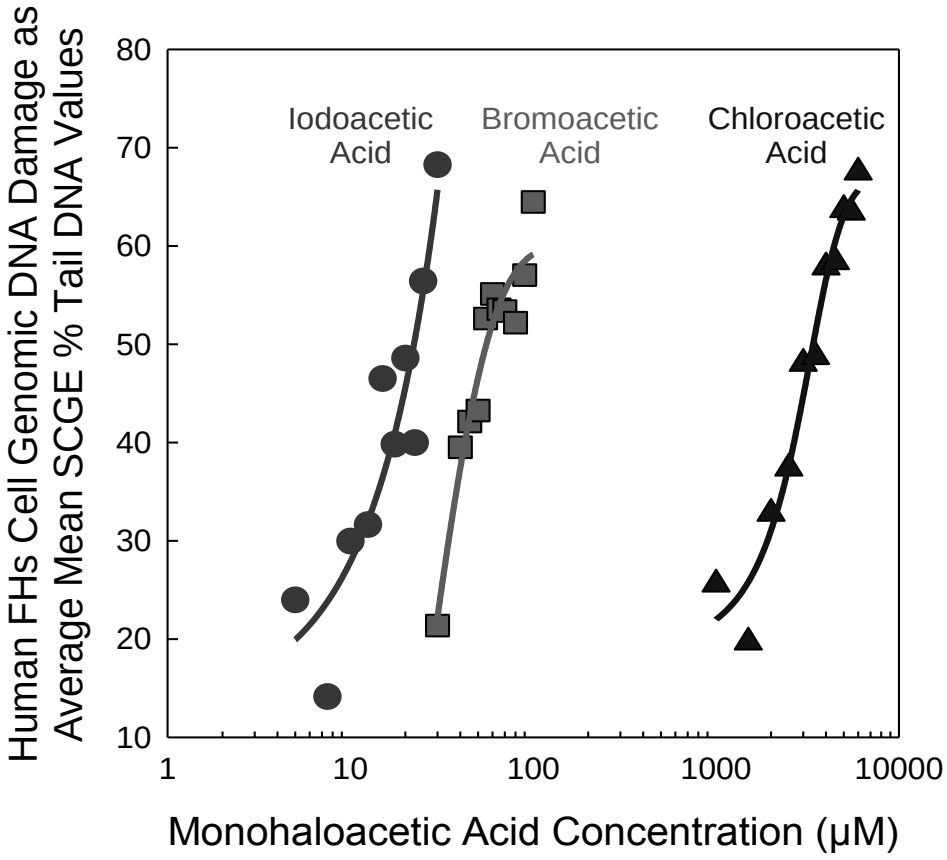

Figure 1.4. SCGE genotoxicity of the monoHAAs in human FHs cells [66].

CHAPTER 2: BIOLOGICAL MECHANISM FOR THE TOXICITY OF HALOACETIC ACID DRINKING WATER DISINFECTION BYPRODUCTS[1]

Preface

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Introduction

Disinfection of drinking water is effective in reducing outbreaks of waterborne disease and was a public health triumph of the twentieth century [1]. However, during the disinfection process, toxic disinfection byproducts (DBPs) are unintentionally formed [2, 3]. Many DBPs are cytotoxic and genotoxic [3, 4]. Recent epidemiological studies demonstrated an association between exposure to DBPs and bladder cancer [5-7] or colon cancer [8]. The U.S. Environmental Protection Agency (U.S. EPA) suggested that 2 to 17% of urinary bladder cancer cases could be induced by DBPs [9]. Recent meta-analyses linked exposure to disinfected water with adverse pregnancy outcomes [10].

The haloacetic acids (HAAs) are the second-most prevalent DBP class generated in disinfected water [11-14]. The HAAs are mutagenic in *Salmonella typhimurium* [15], cytotoxic and mutagenic in Chinese hamster ovary (CHO) K1 cells [16], cytotoxic and genotoxic in CHO AS52 cells [17-19], and in non-transformed human FHs cells [20], cytotoxic but not clastogenic in human TK6 cells [21] and teratogenic [22, 23]. In an effort to limit exposure, the U.S. EPA established a maximum contaminant level of 60 μg/L for the combination of 5 HAAs:

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chloroacetic acid (CAA), dichloroacetic acid, trichloroacetic acid, bromoacetic acid (BAA), and dibromoacetic acid [24].

The HAAs with a single halogen substituent (monoHAAs) include iodoacetic acid (IAA), BAA, and CAA. The level of toxicity induced by the monoHAAs follows the trend of IAA > BAA >> CAA [15, 16, 18-20, 22]. Recent attention has been focused on the occurrence and analytical chemistry of iodoacid DBPs by the Water Research Foundation [25]. The monoHAAs are alkylating agents able to undergo S_N2 reactions (Table 2.1). The relative S_N2 reactivity of the monoHAAs expressed a high correlation with cytotoxicity and genotoxicity in both *S. typhimurium* and mammalian cells [18]. However, the biological molecule(s) with which the HAAs react with and the mechanism(s) by which they induce toxicity remain unclear. The HAAs are considered direct-acting genotoxins because they are mutagenic in *S. typhimurium* without hepatic microsomal (S9) activation [3, 15]. They induce genomic DNA damage and mutagenicity in CHO cells without exogenous cytochrome P_{450} activation [16-18]. However, evidence is emerging that HAAs may not directly interact with genomic DNA. Elevated levels of 8-hydroxydeoxyguanosine in mice treated with chlorinated or brominated HAAs [26-28] suggest that HAA-mediated generation of reactive oxygen species (ROS) are involved in the induction of toxicity and DNA damage. Earlier we demonstrated that catalase and the antioxidant butylated hydroxyanisole significantly reduced IAA-mediated mutagenicity in *S. typhimurium* and genotoxicity in CHO cells, further indicating that a radical species is involved in HAA-induced DNA damage [29].

In neurotoxicology, IAA is employed to induce chemical ischemia (a reduction in the availability of nutrients or oxygen) in neurons by inhibiting glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) leading to cell death [30-33]. The inhibition of GAPDH

blocks glucose metabolism to pyruvate and as a result of disruption of the electron transport chain, in the presence of oxygen, leads to the production of ROS [34]. Recent studies demonstrated that IAA-mediated neuronal damage was caused by ROS generation [30, 32]. Although the biochemical process leading to ROS production is not fully understood, with moderate IAA-mediated blockage of glycolysis, the generation of ROS is linked to calcium (Ca^{2+}) influx through N-methyl-D-aspartate (NMDA) receptors via an excitotoxic mechanism [31]. However, in the same study when neurons were exposed to a higher IAA concentration, an intracellular source of Ca^{2+} was implicated as the NMDA agonist MK-801 did not protect the cells, but BAPTA-AM, an intracellar Ca^{2+} chelator, effectively reduced toxicity [31].

We hypothesize that a common mechanism exists in the induction of HAA-mediated cytotoxicity and genotoxicity observed in bacterial, mammalian and human cells. The objective of this study was to determine whether or not the monoHAAs directly damage genomic DNA, and determine if a cellular process was required for the induction of genotoxicity in CHO cells. We compared the genomic DNA damaging capacity of the monoHAAs under acellular versus cellular conditions. In consort with this hypothesis we quantitatively measured the inhibition kinetics of GAPDH activity induced by the monoHAAs and compared these data with the induction of CHO cell cytotoxicity and genotoxicity.

Materials and Methods

Reagents

General reagents were purchased from Fisher Scientific Co. (Itasca, IL) and Sigma Chemical Co. (St. Louis, MO). BAA and CAA were purchased from Fluka Chemical Co. (Buchs, Switzerland) while IAA was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Glyceraldehyde 3-phosphate was purchased from Sigma-Aldrich (St. Louis, MO). Cell culture F12 medium and fetal bovine serum (FBS) were purchased from Fisher Scientific Co. The HAAs were dissolved in dimethylsulfoxide (DMSO) and stored at −22°C in sealed, sterile glass vials. For the GAPDH activity assay, CAA was dissolved directly into F12 medium while IAA and BAA were diluted from the DMSO stock solutions in Tris-HCl pH 8.5.

Chinese Hamster Ovary Cells

Clone 11-4-8 of the transgenic Chinese hamster ovary (CHO) cell line AS52 was used [4, 35]. The cells were maintained in F12 medium containing 5% FBS, 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA), and 1% glutamine and grown in 100 mm glass culture plates at 37°C in a humidified atmosphere of 5% CO_2.

Acellular Single Cell Gel Electrophoresis

The single cell gel electrophoresis (SCGE or Comet assay) is a sensitive method for measuring genomic DNA damage in individual nuclei from cells [36, 37]. In this study we treated acellular nuclei with monoHAAs to measure the induction of genomic damage without intervening cellular activity. Microgels were prepared using the method described [38]. CHO cells were washed twice with 10 mL Hanks' balanced salt solution (HBSS) and harvested with 1 mL 0.05% trypsin solution in HBSS. Trypsin activity was stopped with 2 mL of F12 +FBS medium and 1 mL of the cell suspension was mixed with 1 mL of 1% low melting point agarose prepared with phosphate buffered saline (PBS). Aliquots (90 µL) of these cell suspensions were placed on clear microscope slides pre-coated with 1% normal melting point agarose prepared with deionized water. The microgels were placed in lysing solution (2.5 M NaCl, 1% sodium

lauryl sarcosinate, 100 mM Na_2EDTA, 10 mM Tris, pH 10 with 1% Triton X-100 and 10% DMSO) overnight at 4°C. Lysed microgels were rinsed 2× with deionized water and submerged in PBS for 5 min. The microgels were treated at pH 7.4 for 2 h at room temperature in Coplin jars with 50 mL of BAA, CAA, or IAA dissolved in PBS. The concurrent positive control was 15% hydrogen peroxide. After treatment, slides were rinsed twice with cold deionized water. The DNA was denatured for 20 min in electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH 13.5). The SCGE microgels were electrophoresed for 40 min at 25 V, 300 mA (0.72 V/cm) at 4°C. After electrophoreses, the microgels were neutralized with 400 mM Tris buffer (pH 7.5), dehydrated in cold methanol for 20 min and dried for 5 min at 50°C. The microgels were stored in the dark at room temperature. Before microscopic analyses, the microgels were hydrated in deionized water at 4°C for 30 min, stained with 65 μL of ethidium bromide (20 μg/mL), and rinsed in cold deionized water. After staining, 25 randomly chosen nuclei per microgel were analyzed with a Zeiss fluorescence microscope (excitation filter of BP 546/10 nm, barrier filter of 590 nm) and a computerized image analysis system (Komet version 3.1, Kinetic Imaging Ltd. Liverpool, UK). The data were automatically transferred to an Excel spreadsheet for statistical analysis. The experiments were repeated 3 times.

Glyceraldehyde-3-phosphate Dehydrogenase Assay

Viable CHO cells or CHO cell homogenates were treated with monoHAAs to determine if they could inhibit the activity of GAPDH. CHO cells were grown to confluence on 60 × 15 mm plastic culture plates, the cells were washed with HBSS and treated with the HAAs in F12 medium in a total volume of 1 mL at 37°C in a humidified atmosphere of 5% CO_2. After treatment (10 - 60 min), the medium was aspirated and the cells were homogenized according to

the procedure published by Chang [39]. CHO cells were washed with cold PBS followed by a wash with cold buffer K (1 mM Tris-HCl, 1 mM EDTA, 1 mM $MgCl_2$, pH 7.6). The cells tumefied at room temperature in buffer K for 2 min. The buffer was aspirated, 200 µL of fresh buffer K (20°C) was added to the dish and the cells were quickly dispatched with a cell scraper. The homogenate was collected into a microfuge tube, mixed, and centrifuged at 16,100 ×g for 2 min to remove cellular debris. The supernatant fluid (CHO cell homogenate) was analyzed for GAPDH activity.

The measurement of GAPDH activity was modified from Ikemoto [40]. A reaction mixture of 0.1 M Tris-HCl (pH 8.5) containing 5 mM KH_2PO_4, 20 mM NaF, 1.7 mM $NaAsO_2$, and 1 mM NAD^+ was prepared. The reaction mixture (976.3 µL) plus 20 µL of cell homogenate served as the blank. The GAPDH reaction was initiated by adding 1 mM glyceraldyde-3-phospate to the reaction mixture (1 mL final volume). GAPDH activity was determined by measuring NADH formation as an increase of absorption at 340 nm every 10 sec for 60 sec using a Beckman DU 7400 UV-Vis spectrometer. Each experiment included 3 monoHAA concentrations and one negative control with 3 replicates for each concentration; experiments were repeated 3 or 4 times. Absorbance values were plotted versus time for each individual experiment and linear regression was conducted. Rates were calculated using the slope of the regression. Average rates were calculated for each concentration at each time point.

Alternatively, untreated CHO cells were grown to confluence and homogenized as above. The reaction mixture was prepared using 20 µL of untreated cell homogenate; the monoHAA diluted in Tris-HCl was added directly to the reaction mixture (final volume 1 mL). GAPDH activity for cellular homogenates was measured using the procedure detailed above. Protein concentration for all homogenates was determined using the microplate-based Bradford assay

according to manufacturer's instructions (Biorad, Hercules CA). GAPDH activity rates were calculated as μMol NADH/min/μg protein.

Results and Discussion

In mammalian systems, the monoHAAs have been shown to be cytotoxic, genotoxic, mutagenic, or teratogenic [16, 17, 20, 22]. In each case the rank order of toxicity was IAA > BAA >> CAA. The mechanisms that lead to this signature of toxic potency signature are not clear.

Direct DNA Damage by MonoHAAs

Among the monoHAAs, a close association exists amongst the halogen leaving group, relative alkylating potential and the induction of adverse biological endpoints measured in a variety of bioassays (Table 2.1) [18]. From these associations we formed a working hypothesis that the monoHAAs interacted directly with DNA to induce genotoxic damage. To test this hypothesis, we compared the induction of genomic DNA damage in treated viable cells versus treated isolated nuclei. Acellular SCGE analyses can determine if an agent directly induces DNA damage without the interaction of cellular targets or metabolism. The SCGE (Comet) assay effectively identifies multiple types of DNA lesions including alkylated bases. Both spontaneous or enzyme catalyzed loss of alkylated bases result in apurinic/apyrimidinic (AP) sites. The AP sites in turn may generate DNA strand breaks by spontaneous cleavage or AP endonuclease-mediated cleavage [41]. Alkylating agents are effective in generating DNA damage in the acellular Comet assay [42-44]. Surprisingly, in the present study the monoHAAs did not induce direct genomic DNA damage in acellular nuclei. No DNA damage was observed for IAA (10 –

500 μM), BAA (10 – 500 μM) or CAA (1 – 10 mM) (Figure 2.1). These data rejected our working hypothesis that the monoHAAs directly interacted with DNA to induce genotoxic damage. The differential responses between the cellular and acellular SCGE analyses (Figure 2.1) demonstrated that the monoHAAs required an interaction with a cellular target or were metabolized to a proximal mutagen in order to inflict genotoxic insult in mammalian cells.

IAA as a Probe of GAPDH Activity

The ability of IAA to inhibit glycolysis has been employed as a model to study the effect of ischemia reperfusion injury in neurons [30, 32, 33, 45-47]. After neuronal IAA exposure, ATP depletion [31, 33, 48], ROS generation [31, 33], phospholipid degradation [33] and neurotoxicity [30, 31, 33, 48] were observed. The attenuation of neurotoxicity by specific antioxidants suggested that ROS play a significant role in cell death [33].

Central to its glycolytic function is a conserved cysteine residue in the active site of GAPDH. This cysteine serves as a nucleophile in the first catalytic step in the conversion of glyceraldehyde-3-phosphate to 1,3-bis-phosphoglycerate. The α-carbon of IAA is a primary alkyl halide and an electrophile due to electron withdrawal from the carbon by the halogen substituent. IAA inhibits GAPDH when the α-carbon undergoes an S_N2 reaction with the nucleophilic thiol group on the catalytic cysteine residue. This results in a carboxymethylated cysteine which irreversibly inhibits the catalytic function of the enzyme. Due to their structural similarity, we predicted that each of the monoHAAs would undergo a similar reaction and irreversibly inhibit GAPDH to varying degrees based on their S_N2 reactivity. The S_N2 reactivity of alkyl halides, including the monoHAAs, is directly related to the leaving efficiency of the halogen substituent, with the rank order of reactivity: IAA > BAA >> CAA. Based on this trend

we predicted that IAA would be the strongest inhibitor of GAPDH, followed by BAA, and CAA would be the weakest (Table 2.1). This prediction formed our new working hypothesis that the monoHAAs induced their relative toxicity profile based on their relative affinity and reactivity in inhibiting GAPDH.

Inhibition of GAPDH Activity by MonoHAAs in CHO Cell Homogenates

Fresh CHO cell homogenates were prepared as described previously and the activity of GAPDH was determined by measuring the formation of NADH. GAPDH activity was expressed as µmol NADH/min/µg protein. All concentrations of the monoHAAs added to the cell homogenates inhibited GAPDH, IAA (10 – 50 µM), BAA (50 – 150 µM) and CAA (5 – 20 mM). The inhibition kinetics of GAPDH is presented in Figure 2.2. The slope for each inhibition curve taken from the linear regression of the data provided the rate of GAPDH inhibition. The reduction of GAPDH activity per µM monoHAA concentration (with their r^2 values) were the following; IAA (-1.04×10^{-2}, $r^2 = 0.99$), BAA (-2.79×10^{-3}, $r^2 = 0.99$), and CAA (-1.81×10^{-5}, $r^2 = 0.99$) (Table 2.1). With CHO cell homogenates the monoHAAs inhibited the activity of GAPDH in a concentration-dependent manner with a rank order of effect expressed as IAA > BAA >> CAA.

Inhibition of GAPDH Activity by MonoHAAs in Treated CHO Cells

To eliminate the possibility that GAPDH inhibition with cell homogenates was artifactual, we treated viable cells and then measured GAPDH activity in the resulting homogenates. CHO cells were treated with IAA (10 – 50 µM), BAA (50 – 150 µM) or CAA (1 – 10 mM) for time periods from 10 to 60 min. Cells were washed, homogenized and the rate of

GAPDH activity was determined as a function of monoHAA treatment concentration and time. Figure 2.3 illustrates the GAPDH inhibition curves induced by BAA treatments of 10, 20, 30 or 60 min. Analyses by linear regression demonstrated that the 20 min treatment time provided the widest range of linear response. The GAPDH rates as a function of monoHAA concentration and treatment times are presented in the Supporting Information. The GAPDH inhibition curves induced by a 20-min treatment of with IAA, BAA or CAA are illustrated in Figure 2.4; the r^2 was 0.93, 0.90 and 0.95, respectively. The reduction of GAPDH activity (μmol NADH/min/μg protein) per μM monoHAA concentration was IAA (-1.58×10^{-2}), BAA (-6.74×10^{-3}) and CAA (-9.44×10^{-5}) (Table 2.1). With intact viable CHO cells the monoHAAs inhibited the activity of GAPDH in a concentration- and time-dependent manner with a rank order of effect expressed as IAA > BAA >> CAA. These data demonstrate that the inhibition of GAPDH was observed with monoHAA-treated viable cells and the results were not an artifact due to homogenation.

Correlation of GAPDH Inhibition with MonoHAA Chemical Characteristics and Toxicity

The objective of this research was to determine a biological mechanism for monoHAA-mediated toxicity. To address this, we conducted multiple correlation analyses of the physiochemical and toxicological characteristics of these monoHAAs using the Pearson's Product Moment test (Table 2.2). Data from this study, from our previous work and data obtained from other studies in the literature for this correlation analyses are presented in Table 2.2. A pattern of toxic potency associated with the halogen leaving group of the monoHAAs was first presented in the pioneering research by Hunter and colleagues [22, 49]. Besides being neuro-teratogens in mice, the HAAs are cytotoxic, genotoxic, and mutagenic in a host of biological assays ranging from bacterial to human cell systems. In earlier work we discovered a

high level of correlation between the cytotoxic and genotoxic responses induced in *S. typhimurium* and CHO cells associated with the α-carbon-halogen dissociation energy, C-X bond length, the relative SN2 alkylating potential and the E_{LUMO} value (lowest unoccupied molecular orbital) of the monoHAAs [18]. In this study we compared the GAPDH inhibition rates induced by the monoHAAs in cell-free homogenates and in treated viable cells with their physiochemical and biological characteristics. The Pearson's Product Moment correlation for the above mentioned physiochemical endpoints and the rates of GAPDH inhibition were highly correlated (Table 2.2). The proclivity of the halogen leaving group and the monoHAA alkylation potential was highly associated with the rates of GAPDH inhibition. Likewise, chronic cytotoxic indexes in *S. typhimurium* ($r = -0.99$) or CHO cells ($r = -0.99$) were highly correlated with GAPDH inhibition (Table 2.2). The levels of mutagenicity induced by the monoHAAs in *S. typhimurium* ($r \geq -0.99$), CHO cell mutagenicity ($r \geq -0.91$), CHO cell genotoxicity ($r \geq -0.97$), and non-transformed human FHs cell genotoxicity ($r \geq -0.99$), were all highly correlated with the rates of GAPDH inhibition (Table 2.2). Finally, mouse *ex vivo* embryo teratogenicity was also highly correlated with monoHAA-mediated GAPDH inhibition ($r \geq -0.98$). These data strongly support the hypothesis that the monoHAAs exert their cytotoxic, genotoxic, mutagenic and teratogenic activities based on their ability to inhibit GAPDH. It has not escaped our attention that direct measurements based on GAPDH inhibition, ROS activity, and adverse biological responses need to be conducted simultaneously in order to rigorously and quantitatively test this hypothesis. Such research is currently being conducted in our laboratory.

While there is a very high correlation between the inhibition of GAPDH and the toxicological endpoints discussed above, a direct biological link between the two continues to be elusive. Although repressing the glycolytic role of GAPDH is central to the induced cytotoxicity,

GAPDH is now known to have an overlay of diverse activities unrelated to its glycolytic function. GAPDH operations include membrane fusion, microtubule bundling, phosphotransferase activity, nuclear RNA export, DNA replication and DNA repair [50], as well as transcriptional control of histone gene expression, nuclear membrane fusion, recognition of mismatched nucleotides, and participation in maintaining telomere structure [51]. Studies on human single-nucleotide polymorphisms of GAPDH demonstrate that these mutations are associated with late-onset Alzheimer's disease [52]. This suggests the possibility that subpopulations may exist with heightened risks to monoHAA-induced ROS. The role of ROS in toxicity is supported by the observations that antioxidants reduce neurotoxicity [31, 33] as well as genotoxicity and mutagenicity [29].

The halogenated acetic acids are a major class of drinking water disinfection byproducts (DBPs) with 5 haloacetic acids regulated by the U.S. EPA. These agents are cytotoxic, genotoxic, mutagenic, and teratogenic. The decreasing toxicity rank order of the monohalogenated acetic acids (monoHAAs) is iodo- > bromo- >> chloroacetic acid. We present data that the monoHAAs inhibit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity in a concentration-dependent manner with the same rank order as above. The rate of inhibition of GAPDH and the toxic potency of the monoHAAs are highly correlated with their alkylating potential and the propensity of the halogen leaving group. This strong association between GAPDH inhibition and the monoHAA toxic potency supports a comprehensive mechanism for the adverse biological effects by this widely occurring class of regulated DBPs.

Supporting Information

Inhibition of GAPDH Activity by Monohaloacetic Acids in Treated CHO Cells

CHO cells were treated with IAA (10 – 50 μM), BAA (50 – 150 μM) or CAA (1 – 10 mM) for time periods from 10 to 60 min. Cells were washed, homogenized and the rate of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was determined as a function of monoHAA treatment concentration and time. GAPDH activity rates were calculated as μMol NADH/min/μg protein. Table 2.3 presents the GAPDH rates of activity as a function of the monohaloacetic acid (monoHAA) concentration and treatment time.

From the data presented in Table 2.3 we determined the best linear responses of monoHAA inhibition of GAPDH were with the 20 min treatment time. These data were used to calculate inhibition slopes that were employed in the Pearson's Product Moment correlation analyses discussed in the paper.

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Tables and Figures

Table 2.1. Chemical and biological characteristics of the monoHAAs.

Mono HAA	C-X	E_{LUMO} (AU)[a]	Bond Length (Å)[b]	Dissociation Energy (kcal/mol)[b]	Relative SN2[b]	Inhibition of GAPDH: CHO Cell Homogenate [c]	Inhibition of GAPDH: Treated CHO Cells [d]
BAA	C–Br	0.111	1.93	65.9	50	-2.79×10^{-3}	-6.74×10^{-3}
CAA	C–Cl	0.126	1.77	78.5	1	-1.81×10^{-5}	-9.44×10^{-5}
IAA	C–I	0.091	2.14	57.4	150-250	-1.04×10^{-2}	-1.58×10^{-2}

[a] Calculated E_{LUMO} (lowest unoccupied molecular orbital) summarized from [53]. [b] Summarized from [54]. [c,d] The average rate of GAPDH inhibition per μM monoHAA concentration derived from the slope of the inhibition curves for each monoHAA presented in Figure 2.2 (treated CHO cell homogenates) or Figure 2.4 (treated intact CHO cells).

Table 2.2. Pearson correlation analyses of physiochemical and toxicological measurements of the monohaloacetic acids.

Physicochemical and toxicological parameters	CHO cell cytotoxic index (*r*)	CHO cell genotoxic index (*r*)	FHs cell genotoxic index (*r*)	*Salmonella* cytotoxic index (*r*)	*Salmonella* mutagenic potency (*r*)	CHO cell mutagenicity index (*r*)	Mouse terato-genicity (*r*)	GAPDH inhibition CHO cell homogenate (*r*) [h]	GAPDH inhibition Treated CHO cells (*r*) [i]
E_{LUMO} [a]	–0.990	–0.996	–0.999	–0.999	–0.999	–0.907	–0.979	0.984	0.999
C-X bond length [b]	0.990	0.997	0.999	0.999	0.999	0.905	0.978	–0.983	–0.999
C-X dissociation energy [b]	–0.945	–0.994	–0.972	–0.974	–0.971	–0.809	–0.922	0.931	0.982
relative SN2 alkylation potential [b]	0.998	0.959	0.988	0.986	0.988	0.973	0.999	–0.999	–0.979
CHO cell cytotoxic index [c]	---	0.976	0.996	0.995	0.996	0.956	0.998	–0.999	–0.990
CHO cell genotoxic index [d]	---	---	0.992	0.993	0.991	0.868	0.959	–0.965	–0.997
FHs human cell genotoxic index [d]	---	---	---	0.999	0.999	0.925	0.987	–0.991	–0.999
Salmonella cytotoxic index [c]	---	---	---	---	0.999	0.921	0.986	–0.989	–0.999
Salmonella mutagenic potency [e]	---	---	---	---	---	0.926	0.988	–0.991	–0.999
CHO cell mutagenicity [f]	---	---	---	---	---	---	0.974	–0.968	–0.906
Mouse teratogenicity [g]	---	---	---	---	---	---	---	–0.999	–0.979

[a] Calculated E_{LUMO} (lowest unoccupied molecular orbital) summarized from [53]. [b] Summarized from [54]. [c] Derived as the reciprocal of the LC_{50} concentration (×constant to generate whole numbers). [d] Derived as the reciprocal of the SCGE genotoxic potency value (×constant to generate whole numbers). [e] Data from [15, 18]. [f] Data from [16]. [g] Data from [22].
[h,i] The average rate of GAPDH inhibition per µM monoHAA concentration derived from the slope of the inhibition curves for each monoHAA presented in Figure 2.2 (treated CHO cell homogenates) or Figure 2.4 (treated intact CHO cells) (this study).

Table 2.3. GAPDH rates in treated CHO cells as a function of monoHAA concentration and exposure times.				
MonoHAA (μM)	Treatment Time and GAPDH Average Rate of Activity[a] ±SE[b]			
	10 min	20 min	30 min	60 min
IAA, 0	1.053 ± 0.086	0.937 ± 0.056	0.989 ± 0.116	0.932 ± 0.178
IAA, 10	0.930 ± 0.074	0.614 ± 0.055	0.540 ± 0.083	0.203 ± 0.026
IAA, 25	0.905 ± 0.213	0.353 ± 0.041	0.219 ± 0.044	0.051 ± 0.006
IAA, 50	0.546 ± 0.134	0.107 ± 0.017	0.047 ± 0.006	0.022 ± 0.004
BAA, 0	1.136 ± 0.187	1.165 ± 0.160	0.990 ± 0.056	0.844 ± 0.048
BAA, 50	0.936 ± 0.078	0.514 ± 0.077	0.242 ± 0.020	0.027 ± 0.005
BAA, 100	0.882 ± 0.076	0.277 ± 0.034	0.065 ± 0.006	0.032 ± 0.010
BAA, 150	0.685 ± 0.122	0.120 ± 0.021	0.030 ± 0.002	0.021 ± 0.002
CAA, 0	0.946 ± 0.045	0.969 ± 0.069	0.917 ± 0.102	0.797 ± 0.009
CAA, 1000	0.936 ± 0.058	0.837 ± 0.055	0.708 ± 0.077	0.431 ± 0.011
CAA, 5000	0.678 ± 0.023	0.303 ± 0.061	0.155 ± 0.026	0.023 ± 0.003
CAA, 10000	0.193 ± 0.013	0.038 ± 0.005	0.032 ± 0.004	0.019 ± 0.006

[a] For each treatment time and monoHAA concentration the GAPDH rate was measured 3× and each experiment was repeated 3 or 4 times. GAPDH activity rates were calculated as μMol NADH/min/μg protein. [b] Standard error of the mean.

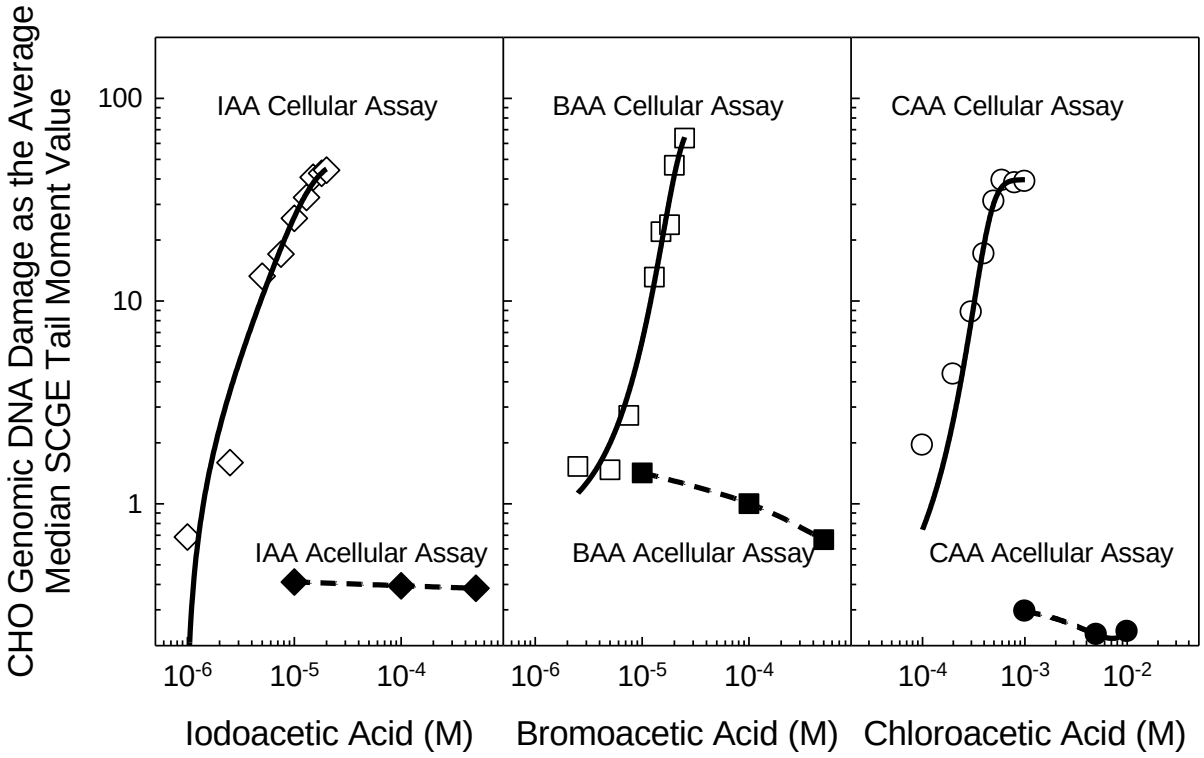

Figure 2.1. Induction of SCGE genotoxicity concentration-response curves for IAA, BAA and CAA in treated viable CHO cells (open symbols) or acellular nuclei (filled symbols). The CHO cell data were from [18].

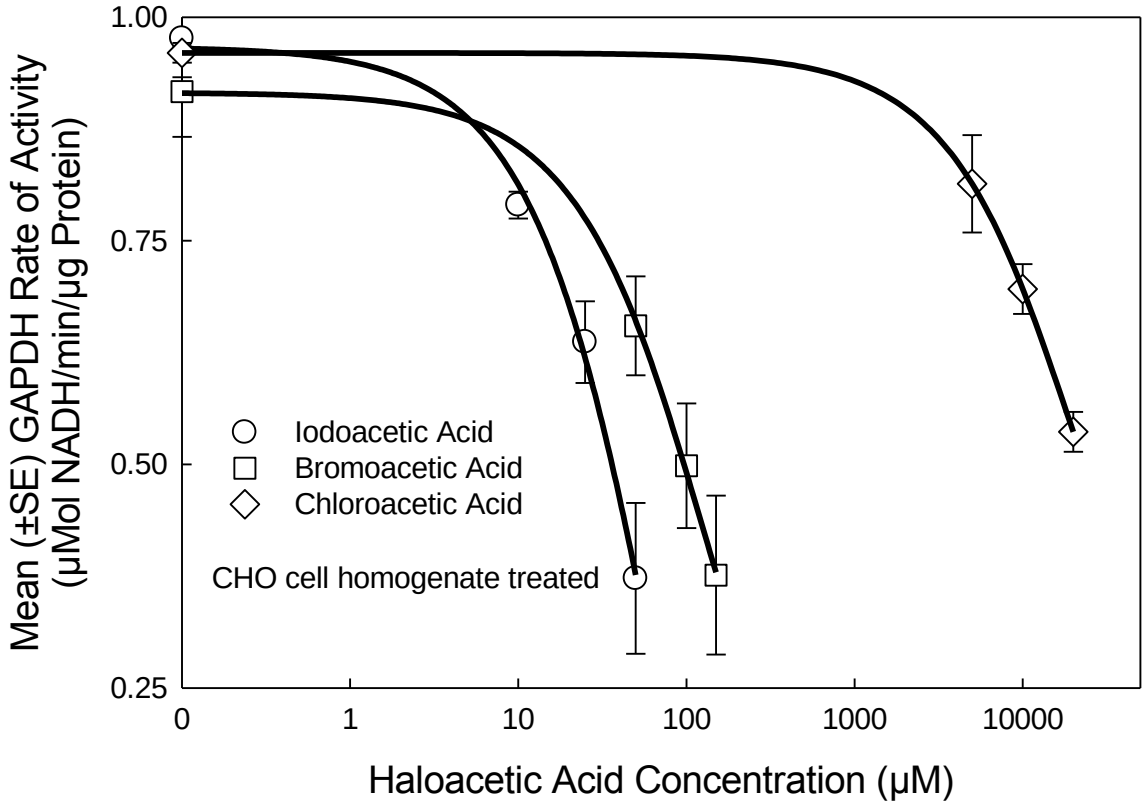

Figure 2.2. GAPDH inhibition curves induced by IAA, BAA, or CAA when added to freshly prepared CHO cell homogenates.

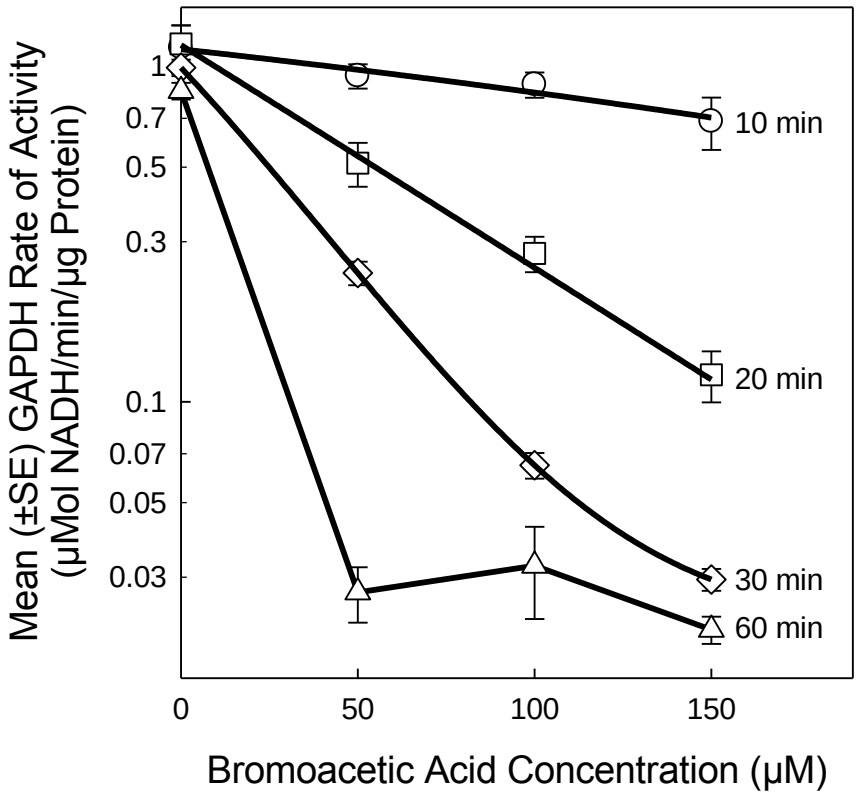

Figure 2.3. GAPDH inhibition curves expressed in CHO cells after treatments with BAA for 10, 20, 30 or 60 min.

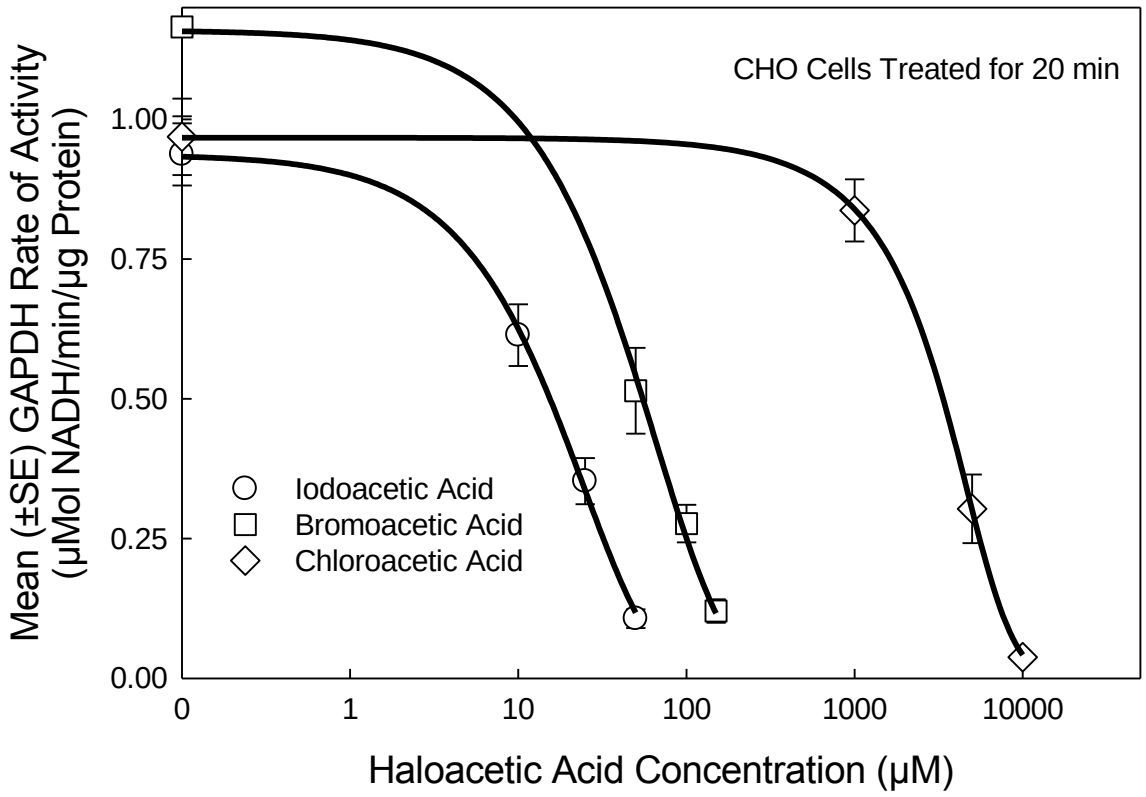

Figure 2.4. GAPDH inhibition curves expressed in CHO cells after treatments with IAA, BAA or CAA for 20 min.

CHAPTER 3: HUMAN CELL TOXICOGENOMIC ANALYSIS LINKS REACTIVE OXYGEN SPECIES TO THE TOXICITY OF MONOHALOACETIC ACID DRINKING WATER DISINFECTION BYPRODUCTS[1]

Preface

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Introduction

Disinfection of drinking water was a major public health achievement of the last century that substantially reduced outbreaks of waterborne disease [1]. During disinfection toxic byproducts (DBPs) are unintentionally generated when the disinfectant reacts with organic matter and inorganic precursors in the source water [2]. Over 600 individual DBPs have been identified in disinfected drinking water [3]; the mixture of DBPs generated varies depending on source water characteristics, disinfection method, DBP precursors and other factors [4, 5]. The United States Environmental Protection Agency (U.S. EPA) regulates 11 DBPs requiring distribution systems to monitor their levels [6]. DBP exposure increased the risk of adverse health outcomes including bladder cancer [7], colorectal cancer [8], and skin cancer [9]. Although DBP exposure during gestation was implicated in adverse pregnancy outcomes

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including fetal growth restriction [10-12] and congenital anomalies these associations are, at present, inconclusive [13].

The U.S. EPA estimated that the population risk to chlorinated water accounted for 2% to 17% of bladder cancer cases in the United States [14]. However, based on animal carcinogenicity data, the regulated DBPs, at levels measured in disinfected water, cannot account for the increased risk of cancer attributed to DBP exposure. This risk is possibly derived from additive or synergic effects of multiple DBPs [15, 16].

The mechanism(s) by which DBPs induce cancer are not understood. Oxidative stress is one mechanism that could explain these adverse health outcomes [17]. Multiple DBPs could contribute to an overall oxidative imbalance within cells or tissues. Biomarkers of oxidative stress have been reported after exposure to individual DBPs including 3-chloro-4-(dichloromethyl)-5-hydroxy-2-(5H)-furanone (MX) [18], bromate [19, 20], di- and trichloroacetate [21, 22], bromodichloroacetate, bromochloroacetate, dibromoacetate [22], chloroacetonitrile [23], dichloroacetonitrile [24], and also organic extracts from disinfected waters [25, 26].

The monohalogenated acetic acid DBPs (monoHAAs), including iodoacetic acid (IAA), bromoacetic acid (BAA), and chloroacetic acid (CAA), are genotoxic in human cells [27] and Chinese hamster ovary (CHO) cells [28-30], and mutagenic in *Salmonella typhimurium* [31], and CHO cells [32]. IAA was a potent inducer of malignant transformation of NIH3T3 cells which were tumorigenic in nude mice [33]. IAA induced reactive oxygen species (ROS), possibly by inhibiting glycolysis [34] causing a reduction of pyruvate, lowering ATP levels and inducing mitochondrial stress. ROS-induced genotoxicity may be the mechanism for the observed malignancy in NIH3T3 cells [33]. The genotoxicity and mutagenicity of IAA were mitigated by

antioxidants [35]. Together these observations suggest that the genotoxicity and mutagenicity of IAA is derived from the generation of ROS. Recently we demonstrated that each monoHAA inhibited glycolysis by inhibiting the enzyme glyceraldehyde phosphate dehydrogenase (GAPDH); the inhibition kinetics strongly correlated with toxicological endpoints, suggesting a common mechanism for these compounds [36, 37].

ROS damage essential biomolecules and lead to cellular dysfunction and disease. Ageing, cancer, atherosclerosis, and neurodegenerative diseases are linked to oxidative stress [38]. Cellular response to oxidative and electrophilic stress is coordinated primarily through the redox sensitive transcription factor, nuclear factor E2-related factor 2 (Nrf2) [39] and antioxidant response elements (AREs), cis-acting factors present in the regulatory regions of ROS/electrophile detoxification genes [40]. This pathway allows cells to respond to oxidative or electrophilic stress by upregulating antioxidant genes, or genes involved in ROS detoxification. ARE-mediated reporter gene assays were used to screen for chemicals that induce oxidative stress [41] and were used as a biomarker of oxidative stress in a recent analysis of a water disinfection system [42].

The objective of this research was to gain additional information on the mechanism(s) of the toxicity of the monoHAAs. We employed an ARE β-lactamase reporter gene assay (ARE-bla assay) to determine if these compounds induce oxidative stress. Subsequently we used toxicogenomic analysis to determine if ROS were generated after monoHAA exposure and monitored specific genes associated with the generation of oxidative stress, or antioxidant response.

Materials and Methods

Reagents

General laboratory chemicals were purchased from Fisher Scientific Co. (Itasca, IL) or Sigma Chemical Co. (St. Louis, MO). Growth medium and fetal bovine serum (FBS) were purchased from Hyclone Laboratories (Logan, UT). Human epidermal growth factor (EGF) was purchased from Sigma Chemical Co., BAA and CAA were purchased from Fluka Chemical Co. (St. Louis, MO) IAA was purchased from Aldrich Chemical Co (St. Louis, MO).

ARE-bla HepG2 Reporter Gene Assay

The CellSensor® ARE-bla HepG2 cell line (Life Technologies, Madison, WI) contains a β-lactamase reporter gene under control of the Antioxidant Response Element (ARE) stably integrated into HepG2 cells. Cells were maintained in DMEM with glutamax (Life Technologies) supplemented with 10% dialyzed FBS, 0.1 mM NEAA, 25 mM HEPES, 100U/mL Penn-strep and 5 μg/mL blasticidin at 37°C and 5% CO_2. Cells were harvested using 0.25% trypsin, centrifuged, and suspended in assay medium (DMEM with glutamax supplemented with 1% dialyzed FBS, 0.1 mM NEAA, 25 mM HEPES and 100U/mL Penn-strep). Cells were plated at 2000/well/5 μL in 1536 well black-clear bottom plates (Greiner Bio-One North America, Monroe, NC) and incubated at 37°C, 5% CO_2 for 5 h. Twenty-three nL of compounds or a positive control, β-napthoflavone, were transferred to each well using a pintool (Kalypsys, San Diego, CA). Cells were incubated at 37°C, 5% CO_2 for 16 h. After the incubation period, 1 μL of CCF4 dye (Life Technologies) was added to each well and the plate was incubated at room

temperature for 2 h. Fluorescence intensity at 460 and 530 nm emissions was measure at 405 nm excitation by an Envision plate reader (PerkinElmer, Boston, MA) followed by an addition of 4 μL/well of cell viability reagent (CellTiter-Glo, Promega, Madison, WI). The plates were incubated for 30 min at room temperature and luminescence was read using a ViewLux plate reader (Perkin-Elmer). For the ARE-bla assay, data were expressed as the ratio of the 460/530 emission values, normalized to the positive control response (23 μM β-napthoflavone, 100%). For cytotoxicity, data were normalized to 100% for the negative control, (DMSO) and to 0% for the basal cytotoxic control (92 μM tetraoctyl ammonium bromide).

Human Intestinal Epithelial Cells

Nontransformed human intestinal cells, line FHs 74 Int, were purchased from American Tissue Culture Collection (Manassass, VA.) Cells were received at passage 12, and used until passage 17 or 18. Cells were maintained in Hybri-care 46X medium supplemented with 10% FBS, 1% antibiotic (10 units/mL penicillin G sodium, 10 μg/mL streptomycin sulfate, 25 μg/mL amphotericin B, 0.85% saline), and 30 ng/mL human epidermal growth factor at 37°C in a humidified atmosphere of 5% CO_2.

Cell Viability

To reduce artifactual transcriptome alterations derived from cell death, monoHAA concentrations were selected that produced equal genomic DNA damage in FHs 74 Int cells with minimal effect on cell viability [27]. Cell viability was measured both immediately and 24 h after HAA exposure as previously reported [27].

RNA Isolation, cDNA Synthesis, Real Time Quantitative PCR Analysis

Four days prior to treatment 4×10^5 FHs 74 Int cells were seeded into the wells of a 6 well plate. Prior to exposure the cells were washed twice in Hank's balanced salt solution (HBSS). HAAs were added in culture medium without FBS. After either 30 min or 4 h exposure, the cells were washed and harvested with trypsin (0.05%) and centrifuged. Four h was chosen as the time period for late expressing genes since this time was standardized for the DBPs to determine the equivalent levels of DNA damage [27, 30, 43]. For early gene expression, we chose 30 min treatments because this was the earliest interval that we could technically repeat the procedure and demonstrate consistent gene expression. Aliquots of the cell suspension were retained for analyses of acute toxicity and genomic DNA damage with the single cell gel electrophoresis assay. After the supernatant was removed, RNA was isolated using a Qiagen RNeasy Mini Kit (Valencia, CA) following the manufacturers protocol. RNA integrity was determined using an Agilent 2100 Bioanalyzer (Santa Clara, CA). RNA integrity (RIN) numbers were determined for RNA from each exposure group and their concurrent negative controls. cDNA was prepared from RNA using SuperArray RT^2 PCR Array First Strand Kit (SA Biosciences, Frederick, MD), cDNA samples were diluted in nuclease free water and stored at −20°C. Transcriptome profiles were analyzed using an Oxidative Stress and Antioxidant Defense pathway specific PCR array (SuperArray PAHs-065-24). A list of genes included in this PCR array is presented within their functional gene groupings (Table 3.4). Real-time PCR analysis was conducted using a two-step cycling program on a Stratagene Mx3000p thermocycler. Quality controls measuring genomic DNA contamination, reverse transcription, and PCR amplification efficiencies were included and

analyzed. Because SYBR Green technology was utilized, melting curves were investigated for each gene analyzed. Wells with multiple amplification products were excluded.

Safety and Data Handling

Manipulations of toxic chemicals were conducted in certified biological/chemical stage-2 safety hoods. Average Ct values for each gene were calculated against the average of 5 housekeeping genes (B2M, HPRT1, RPL13A, GAPDH and ACTB) using the SA Biosciences PCR analysis software RT^2 Profiler Data Analysis Template. This software also estimated fold changes using the ΔΔCt method comparing treated cells to concurrent negative controls. The RankProd algorithm from the R Bioconductor package was used to test significance [44], with $P \leq 0.05$ considered significant. The raw and normalized data are available in the Gene Expression Omnibus (GEO) database [45] under the NCBI tracking system GSE49698 series accession number.

Results and Discussion

ARE-dependent gene expression in a reporter gene assay demonstrated a strong response after cells were exposed to the monoHAAs. These responses implicated the generation of ROS as a function of monoHAA concentration. Previous studies suggested that the monoHAAs induced their cytotoxicity and genotoxicity by generating ROS based on the inhibition of GAPDH [36], the repression of ATP levels, pyruvate remediation of cell stress and genotoxicity [37], repression of genotoxicity by radical scavengers and antioxidants [35, 46], and the direct measurement of cellular ROS after monoHAA treatment [46]. We proposed that each of the monoHAAs share a similar ROS-mediated mechanism of toxicity and tested this hypothesis using an ARE reporter assay and toxicogenomic analyses.

Toxicogenomics measured as toxicant-induced modulation of gene expression is a powerful analytical tool for cell stress. When compared to concurrent control transcriptome profiles, metabolic pathways involved in the cellular responses to toxic agents can be identified and provide insights into the biological mechanisms of toxicity. Many *in vitro* toxicogenomic studies employ human or mammalian tumor cell lines because of their ease of growth and in some cases cells are exposed to cytotoxic concentrations to observe effects on gene expression. Tumor cell lines inherently exhibit aberrant gene expression. With cytotoxic concentrations, transcript profiles will reflect those of dead or dying cells. We avoided these complications by using nontransformed human cells, concurrent negative controls at each treatment time and noncytotoxic concentrations of each monoHAA. An additional concern is that much of the gene expression literature is based on whole genome arrays without qRT-PCR confirmation. Our experimental design to determine transcriptome profiles of the monoHAAs was based on the direct use of PCR gene arrays. Our previous data argued that the monoHAAs were genotoxic due to their ability to induce ROS. This was the hypothesis that we tested using a ROS-responsive gene array.

Besides the employment of a gene array for oxidative stress we used ARE transcription as an independent indicator. Briefly, the ARE-bla HepG2 cell line contains three stably integrated copies of the ARE derived from the human nicotinamide adenine dinucleotide phosphate (NADPH) quinone oxidoreductase 1 gene (NQO1) that drive the expression of a β-lactamase reporter gene [41]. Oxidative stress increases Nrf2 dependent and independent ARE-driven transcription of β-lactamase [41]. β-lactamase cleaves an exogenous substrate (coumarin) reducing the fluorescence resonance energy transfer (FRET) signal emitted by the intact substrate, while increasing the emission from the cleaved substrate. ARE-driven transcription of

β-lactamase is quantified by measuring the emission ratio of 460:530 nm, the emission of the β-lactamase cleaved coumarin over the emission of the intact substrate. Increased production of ARE-linked β-lactamase shifts the fluorescence towards 460 nm and is indicative of oxidative stress. Each monoHAA generated concentration-dependent increases in ARE-dependent transcription of β-lactamase, as evidenced by the shift in fluorescence towards 460 nm; the data, normalized to the positive control (23 μM β-napthoflavone) are displayed in Figure 3.1. These data demonstrated that the monoHAAs induced the activation of the antioxidant response pathway suggesting the generation of oxidative stress by these DBPs. The EC_{50} values of IAA, BAA or CAA that induced ARE-dependent β-lactamase activity were 1.17, 3.16 and 16.2 μM, respectively. Using the $EC_{IR1.5}$ metric (i.e. a 1.5 fold increase over the baseline) [47] the values for IAA, BAA or CAA were 0.99, 1.96 and 8.65 μM, respectively. A Pearson Product Moment Correlation analysis demonstrated that ARE expression by the monoHAAs in human HepG2 cells and genomic DNA damage induced by the monoHAAs in human FHs 74 Int cells [27] were highly correlated ($r = 0.99$; $P \leq 0.07$). The response pattern of IAA > BAA >> CAA, observed in previous measurements of monoHAA-mediated toxicity, was conserved.

ARE-dependent gene expression in a reporter gene assay demonstrated a strong response after cells were exposed to the monoHAAs implicating the generation of ROS as a function of monoHAA concentration. These responses also indicated that transcription rates of genes regulated by ARE could be altered by monoHAA exposure. Transcription factors activated by oxidative stress can bind to AREs and modify transcription of genes coding for proteins with antioxidant or ROS detoxification functions. While a review of ARE responsive genes is beyond the scope of this paper, proteins acting in the glutathione/glutathione reductase (GSH/GSR), thioredoxin/thioredoxin reductase (Txn/Txnrd) pathways, as well as superoxide dismutases

(SOD), and catalase among others are all regulated by AREs [48]. The Human Oxidative Stress and Antioxidant Defense pathway specific PCR array contained members of each of these antioxidant pathways, along with additional genes involved with the production or metabolism of ROS and oxidative stress responsive genes. The oxidative stress responsive genes in the array were used collectively as an indicator of oxidative stress. Because it included several ARE-responsive genes, this array served as an additional measure of the effect of monoHAAs on ARE-driven transcription. This analysis also allowed us to evaluate the effect of the monoHAAs on 83 specific genes and several pro-, or antioxidant pathways, at the transcriptional level.

Toxicogenomic analysis quantifies mRNA and measures changes in transcription patterns in response to toxic insult. We exposed non-transformed human FHs 74 Int cells, to 22 μM IAA, 57 μM BAA, or 3.42 mM CAA, concentrations that generated equivalent genomic genotoxicity (approximately 50% tail DNA values measured by single cell gel electrophoresis) without causing significant acute cytotoxicity [27]. This experimental design reduced artifactual changes due to transcriptome profiles generated from dead or dying cells, or from altered gene expression/regulation that might be associated with cancer cell lines. Two exposure times, 30 min and 4 h, were chosen to evaluate early and late changes in transcription patterns. Each treatment was compared to a concurrent negative control. One gene, neutrophil cytosolic factor 1 (NCF1), was removed from analysis because multiple amplification products, as evidenced by multiple peaks in the melting point analysis, were detected.

Each of the monoHAAs altered transcription for multiple genes at both exposure times. Genes with statistically significant alterations in transcription (positive calls) are listed in Table 3.1 (30 min exposure) and Table 3.2 (4 h exposure). Positive calls were sorted into functional gene groupings including: glutathione peroxidases (GPx), peroxiredoxins (TPx), other

peroxidases, other antioxidants, superoxide dismutases (SOD), other genes involved in superoxide metabolism, other genes involved in ROS metabolism, and oxidative stress responsive genes.

After a 30 min exposure to individual monoHAAs, 31 of the 83 genes assayed exhibited significantly altered expression levels, as compared to negative controls, with a total of 45 positive calls amongst all treated groups (Table 3.1, Figure 3.2). There was considerable overlap of altered gene expression, with 14 of the 31 (45.2%) positive calls generated by two monoHAAs. None of the altered gene expressions were shared by all 3 monoHAAs. Of the 45 positive calls, 26 (57.8%) were downregulated and 19 (42.2%) were upregulated (Table 3.1).

For the 4 h exposure, a total of 47 gene expression levels were altered amongst all treatment groups, with transcription levels of 28 genes significantly altered (Table 3.2, Figure 3.2). Of the genes with significantly modified transcription levels, 11 (37.9%) were altered by 2 monoHAAs, and 4 (13.8%) were altered by all three monoHAAs. Of the 47 positive calls, 26 (55.3%) were downregulated and 21 (44.7%) were upregulated (Table 3.2).

For each monoHAA exposure, at both time points, multiple oxidative stress responsive genes showed altered transcription patterns (Figures 3.2 and 3.3). Taking these genes collectively as an indicator, our hypothesis is supported that cells exposed to monoHAAs are responding to oxidative stress.

Several genes involved in antioxidant defense or detoxification are regulated by the Nrf2/ARE system at the transcription level [48]. Analysis of the transcriptome profiles showed that ARE-responsive genes exhibited modified transcription levels after monoHAA challenge, although no clear pattern of regulation emerged when all ARE-responsive genes were considered. Some of the ARE-responsive genes were upregulated: epoxide hydrolase (EPHX2)

after 30 min exposure to BAA or CAA, sulfiredoxin (SRXN1) after 4 h exposures to IAA, BAA or CAA and thioredoxin reductase 1 (TXNRD1) after 4 h exposure to BAA or CAA. Other ARE-responsive genes were downregulated after 30 min: TXNRD1 with CAA, glutathione reductase (GSR) with IAA or BAA, as well as after 4 h: peroxiredoxin 3 (PRDX3) and GSR. ARE-regulated genes including SOD, and those peroxiredoxins and glutathione peroxidases not included in Tables 3.1 and 3.2 did not show altered transcription patterns after monoHAA exposures. These findings suggest that while monoHAAs activate ARE-driven transcription in the ARE-bla reporter system, the regulation of specific genes within the antioxidant pathways of the FHs cell line includes additional layers of complexity.

Within the GSH/GSR pathway, GPX2, GPX6 and GSR genes were downregulated (Tables 3.1 and 3.2). None of the SOD genes or catalase displayed altered transcription after monoHAA exposures at either time point. These data along with the altered transcription of SRXN1 and TXNRD1 suggested that peroxiredoxins (Prx) were the main target for ROS generated during monoHAA challenge.

Prx proteins reduce hydrogen peroxide, peroxynitrite, and lipid peroxides. Each of the 6 mammalian PRX genes was included in the gene array, however, only PRDX3 and PRDX4 were modified by monoHAA exposure. Peroxiredoxins contain 1 (Prx6) or 2 (Prx1-5) catalytic cysteine residue(s) that is/are oxidized during their peroxidase activity. TXNRD1 and SRXN1 encode proteins capable of reducing oxidized Prxs. Txnrd1 acts indirectly by regenerating reduced Txn after it has been oxidized while reducing Prx [49]. Sulfiredoxin directly reduces Prx1-4, the so-called typical 2 cysteine Prx [50]. Both TXNRD1 and SRXN1 were upregulated by BAA and CAA or all monoHAAs, respectively, after 4 h. However, none of the PRX genes were upregulated at any time point. PRDX3, was downregulated after 4 h exposure to BAA and

IAA. The significance of the downregulation was unclear. However, post-transcriptional modifications that inactivate Prx provide localized increases in H_2O_2 concentration important for modifying signaling cascades. Prx3 specifically functions within the mitochondria to regulate apoptosis [51]. Prx proteins were modulated post-transcriptionally to allow local increases of H_2O_2 to modify growth factor signaling [52]. Metabolites of arachidonic acid (AA) metabolism oxidized and inhibited Prx1-3, suggesting a role in signaling pathways [53].

AA is liberated from membranes via calcium dependent cytosolic phospholipase A2 ($cPla_2$) and is metabolized through cyclooxygenase (COX) or lipoxygenase (LOX) pathways yielding eicosanoid signaling molecules [54]. Both COX [55] and LOX [56] pathways generate superoxide and lipid peroxides as byproducts of AA metabolism. Free AA was measured in the medium of IAA treated neurons [57]. PLA_2 inhibitors [58], antioxidants [34] and the calcium chelator BAPTA-AM [34] each reduced IAA-mediated toxicity. These results suggest a process involving increased intracellular [Ca^{2+}], activation of $cPla_2$, and ROS production that is involved in IAA-generated neurotoxicity. In this study we found each monoHAA exposure altered mRNA levels of COX to a greater degree than LOX enzymes. ALOX12 mRNA was decreased after BAA (30 min and 4 h) or CAA (30 min) exposures. Conversely, PTGS2 (COX-2) was downregulated by BAA after 30 min but upregulated by IAA at 30 min, and upregulated by all three monoHAAs after 4 h exposures (Tables 3.1 and 3.2). COX-2 exhibited the largest increase in each of the 4 h treatment groups, with a >32× increase in BAA treated cells.

In addition to the generation of ROS as a byproduct of AA metabolism, the upregulation of COX-2 suggests a possible inflammatory response which may impact human health. Chen et al showed that CAA activated the stress associated mitogen activated protein kinase (MAPK) p38 pathway via oxidative stress [59]. Activated p38 phosphorylates and inactivates

tristetraproline preventing the degradation of both COX-2 and dual specific phosphatase 1 (DUSP1) mRNA [60]. Our data demonstrated that 4 h exposures to BAA or IAA increased COX-2 and DUSP1 mRNA levels, suggesting that activation of the p38 pathway may be a common response to monoHAA exposure. Cox-2 and p38 play critical roles in inflammation. Previous toxicogenomic analyses focused on DNA damage and repair pathways demonstrated that a 4 h exposure to IAA modified genes within the FcεRI receptor pathway [27]. FcεRI functions in phagocytosis and production of pro-inflammatory cytokines [61].

Inflammation has been linked to all phases of cancer [62]. The link between COX-2 and bladder and colon cancers, the cancers most commonly associated with DBP exposures [7, 8], was well documented. Prostaglandin E2, a byproduct of COX-2 mediated AA metabolism, increased colon cancer growth by increasing β-catenin signaling [63]. Furthermore, COX-2 inhibitors reduced growth of HT-29 and Caco-2 colon cancer cell lines [64], and inhibited haematogenous metastasis of colon cancer in mice [65]. COX-2 was detected in transitional cell carcinomas [66], and also squamous cell carcinomas [67] of the urinary bladder. Selective inhibitors of COX-2 reduced incidence of bladder cancer in male rats exposed to *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine [68, 69]. AA metabolism also inhibited the tumor suppressor PTEN [54]. Although further studies are needed to verify an inflammatory effect *in vivo*, the data presented here could have important implications in linking DBP exposure with associated adverse health outcomes.

While previous studies showed ROS resulted from IAA exposures, the source(s) were not defined. Alterations in transcriptome profiles could provide insight into mechanisms by which ROS were generated during monoHAA exposure. Pro-oxidant encoding genes upregulated by two or more monoHAA exposures were considered potential sources of ROS. In addition to ROS

generated through COX-2 catalyzed metabolism of AA, two additional sources of ROS were identified in the hypohalous acid generating peroxidases (myeloperoxidase (MPO) and lactoperoxidase (LPO)) and NADPH dependent oxidase 5 (NOX5) (Tables 3.1 and 3.2).

MPO was upregulated by a 30 min exposure to BAA or CAA; LPO was upregulated by CAA or IAA (Table 3.1). NOX5 was upregulated by CAA after 30 min, and by each of the monoHAAs after 4 h exposure (Tables 3.1 and 3.2). Noxs generate superoxide anion ($O_2^{-}\cdot$) by transferring an electron from NADPH to molecular oxygen. NOXs work in tandem with Lpo, or Mpo proteins in the epithelial mucosa [70] or phagocytes [71], respectively, to generate hypohalous acids (HOX, where X represents I, Br, Cl, or thiocyanate (SCN)) for host defense. Nox enzymes were activated by $cPla_2$ [72], and Nox5 is Ca^{2+}-dependent [73]. Additional research is needed to evaluate the source(s) of ROS generated during monoHAA exposure and their contributions to the overall oxidative imbalance.

In summary, we employed the ARE-bla HepG2 reporter cell line as a biomarker for oxidative stress and observed an increase in ARE driven β-lactamase transcription in cells treated with each of the monoHAAs. Each exposure caused oxidative stress in the reporter cell line. The rank order IAA > BAA >> CAA found in the activation of the ARE response pathway is consistent with toxicological endpoints previously measured [27-29, 31, 32, 36, 37, 74]. Additionally we exposed non-transformed human embryonic intestinal epithelial cells (line FHs 74 Int) to equally genotoxic, but non-cytotoxic levels of the monoHAAs, IAA, BAA, and CAA for 30 min or 4 h and investigated the transcription of 83 genes involved in oxidative stress and antioxidant response. Each monoHAA at both exposure times caused significant changes in transcriptome profiles. The high number of modified oxidative stress responsive genes confirmed previous reports that IAA and CAA induced oxidative stress, and demonstrated that BAA shared

this mechanism. The gene array data indicated a cellular response to oxidized Prx proteins, in that SRXN1 and TRXND1 were upregulated, whereas GSR was downregulated, and other antioxidant pathways were unchanged. The data also suggest a possible link between monoHAA exposure, inflammation, and cancer, with COX-2 connecting these three phenomena. The data indicated three possible sources of ROS, (1) HOX derived from peroxidase enzymes such as Mpo or Lpo, (2) $O_2^{-}\cdot$ derived from Nox5, and (3) ROS derived from the metabolism of AA via COX-2.

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Supporting Information

MonoHAA Treatment for Toxicogenomic Analyses, RNA Isolation and Purification

Four days prior to treatment, 4×10^5 FHs 74 Int cells were seeded in each well in six-well plates. FhS cells were treated with a 30 min or 4 h exposure to 22 µM IAA, 57 µM BAA or 3.42 mM CAA. The distribution of genomic DNA damage for the negative control and for cells treated with IAA, BAA or CAA is presented in Figure 3.4. FHs cells were washed twice with

HBSS, harvested, and centrifuged at 300× g for 5 min. An aliquot of each cell suspension was retained prior to centrifugation for acute cytotoxicity and SCGE analyses. The supernatant was removed and RNA isolated using a Qiagen RNeasy Mini Kit. DNase treatment of the RNA samples was conducted using Ambion DNA-free DNase and the RNA concentrations were determined using the NanoDrop 1000 from Thermo Fisher Scientific. The resulting genomic DNA-free RNA was concentrated by vacuum centrifugation for 15 min using a Speedvac system. RNA quantity was determined using the Agilent 2100 Bioanalyzer. RNA Integrity Numbers (RIN) were in the range from 8.2 to 9.9 (RNA quality for microarray analysis must have RIN values greater than 7) [75]. High quality RNA is essential for qRT-PCR arrays. Figure 3.5 presents the nanodrop, electrophoresis of the RNA isolated from the control and IAA-treated FHs cells. The capillary electrophoresis analysis of the RNA isolated from each FHs cell sample in the IAA experiments is illustrated in Figure 3.6. The RNA concentration and purity (RIN values) for each monoHAA treatment group is presented in Table 3.3.

cDNA Synthesis

cDNAs were synthesized using the SuperArray RT^2 PCR Array First Strand Kit (Frederick, MD). RNA samples were diluted to a constant concentration for each monoHAA exposure. One µL of the P2 enzyme, from the SuperArray RT^2 PCR Array First Strand Kit, was added to the nuclease-free PCR tube containing the diluted RNA.

Using a MJ Research PTC-100 programmable thermocycler the annealing reaction was conducted at 70°C for 3 min and held on ice. The RT cocktail was prepared by mixing 10 µL of the annealing mixture with 10 µL of the RT cocktail. This mixture was incubated at 37°C for 60 min and heated to 95°C for 5 min to hydrolyze the RNA and inactivate the reverse transcriptase.

The finished reaction was held on ice. After cDNA synthesis, the samples were diluted with 91 µL of nuclease free water and stored at −20°C.

qRT-PCR array

An Oxidative Stress and Antioxidant Defense pathway specific PCR array (SuperArray PAHs-065-24) induced by the monoHAAs [76]. The human genes evaluated for their expression are listed in Table 3.4. An aliquot of the diluted first strand synthesis reaction was added to the SuperArray RT^2 Real-Time SYBR Green/ROX PCR master mix and nuclease-free H_2O. This cDNA/master mix cocktail was transferred to a sterile, nuclease free reservoir and 25 µL were placed into each well of a pathway specific qRT-PCR array. Optical cap strips were tightly placed onto each column and the microplate was centrifuged. Quantitative real-time PCR analysis was conducted using a two-step cycling program on a Stratagene Mx3000p thermocycler. For each array we analyzed quality controls that measured genomic DNA contamination, reverse transcription efficiency, and PCR amplification efficiencies and these quality control parameters were within accepted limits.

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Tables and Figures

Table 3.1. Gene Expression Level Changes vs. Concurrent Negative Controls after 30 min MonoHAA Exposure.							
		IAA		BAA		CAA	
Gene Symbol	Functional Gene Group(s)	Fold Change	*P*-value	Fold Change	*P*-value	Fold Change	*P*-value
ALOX12	Superoxide Metabolism			-2.286	0.015	-1.972	0.013
APOE	Antioxidant, Oxidative Stress Responsive	-1.725	0.040				
CCL5	Oxidative Stress Responsive	3.588	0.001	2.424	0.004		
CYBA	Superoxide Metabolism					-1.519	0.045
CYGB	Peroxidase, Oxidative Stress Responsive	-1.941	0.038				
DGKK	Oxidative Stress Responsive			2.057	0.008		
DHCR24	Oxidative Stress Responsive	-1.832	0.050				
DUOX1	Peroxidase, Superoxide Metabolism, Oxidative Stress Responsive			-2.608	0.004	-2.334	0.001
EPHX2	ROS Metabolism			2.856	0.001	8.041	< 0.001
EPX	Peroxidase, Oxidative Stress Responsive					-1.857	0.017
GPX2	Glutathione Peroxidase, Oxidative Stress Responsive			-4.396	< 0.001		
GSR	Antioxidant	-4.521	<0.001	-3.433	0.007		
KRT1	Oxidative Stress Responsive					3.549	0.008
LPO	Peroxidase, Oxidative Stress Responsive	1.823	0.034			3.919	0.022
MBL2	Oxidative Stress Responsive					2.702	0.038
MPO	Peroxidase, Oxidative Stress Responsive			1.516	0.035	2.509	0.004
NOS2A	Superoxide Metabolism					7.000	0.002
NOX5	Superoxide Metabolism	-1.782	0.042			3.901	0.004
PIP3-E	Peroxidase, Oxidative Stress Responsive	1.977	0.046	1.386	0.034		
PNKP	Oxidative Stress Responsive	-2.033	0.023			-1.701	0.011

Table 3.1. (cont.)							
PTGS2 (COX-2)	Peroxidase	5.122	< 0.001	-2.410	0.004		
PXDNL	Peroxidase	-3.182	0.012				
RNF7	Oxidative Stress Responsive	1.803	0.019				
SCARA3	Oxidative Stress Responsive					-1.840	0.013
SFTPD	ROS Metabolism					1.973	0.017
SGK2	Oxidative Stress Responsive			-2.422	0.007		
SIRT2	Oxidative Stress Responsive	-1.782	0.039			-1.616	0.017
STK25	Oxidative Stress Responsive			-1.990	0.038		
TPO	Peroxidase, Oxidative Stress Responsive			-2.339	0.012	-2.377	0.002
TXNDC2	Antioxidant	3.775	0.001			-1.931	0.022
TXNRD1	Antioxidant					-1.543	0.049

Table 3.2. Gene Expression Level Changes vs. Concurrent Negative Controls after 4 h MonoHAA Exposure.							
		IAA		BAA		CAA	
Gene Symbol	Functional Gene Group(s)	Fold Change	*P*-value	Fold Change	*P*-value	Fold Change	*P*-value
ALB	Antioxidant	1.785	0.011				
ALOX12	Superoxide Metabolism			-3.125	0.020		
ANGPT7L	Oxidative Stress Responsive			-5.900	0.001		
AOX1	ROS Metabolism	1.785	0.017				
CCL5	Oxidative Stress Responsive	1.981	0.011			-2.074	0.018
DUOX1	Peroxidase, Superoxide Metabolism, Oxidative Stress Responsive	-11.610	< 0.001	-2.463	0.044		
DUSP1	Oxidative Stress Responsive	1.994	0.009	14.117	< 0.001		
EPHX2	ROS Metabolism					-2.737	0.004
EPX	Peroxidase, Oxidative Stress Responsive	-2.331	0.034	-3.993	0.004	-1.810	0.021
GLRX2	Oxidative Stress Responsive			-4.837	0.006		
GPR156	Peroxidase, Oxidative Stress Responsive			-2.504	0.032		
GPX2	Glutathione Peroxidase, Oxidative Stress Responsive			-2.957	0.019		
GPX6	Glutathione Peroxidase, Oxidative Stress Responsive					-2.377	0.016
GSR	Antioxidant	-2.683	0.012	-2.671	0.037		
MTL5	Oxidative Stress Responsive	1.949	0.006				
NOS2A	Superoxide Metabolism	-4.020	0.002			-1.931	0.041
NOX5	Superoxide Metabolism	1.976	0.008	2.323	0.016	3.830	0.001
PIP3-E	Peroxidase, Oxidative Stress Responsive	-4.058	0.003			4.694	0.000
PRDX3	Peroxiredoxins			-3.040	0.014	-1.823	0.040
PRDX4	Peroxiredoxins					-1.985	0.032
PTGS2 (COX-2)	Peroxidase	5.108	< 0.001	32.282	< 0.001	8.153	0.000
PXDNL	Peroxidase	-3.326	0.009			1.845	0.045
SFTPD	ROS Metabolism			-3.476	0.009		
SGK2	Oxidative Stress Responsive	1.433	0.033	3.587	0.007		
SRXN1	Antioxidant, Oxidative Stress Responsive	2.366	0.002	4.477	0.001	2.320	0.007

Table 3.2. (cont.)							
TPO	Peroxidase, Oxidative Stress Responsive			-3.866	0.004		
TTN	Peroxidase, Oxidative Stress Responsive	-3.665	0.003	-5.467	0.001		
TXNRD1	Antioxidant			1.918	0.036	2.341	0.008

Table 3.3. RNA concentration and purity for each monoHAA treatment group		
monoHAA Treatment Group	RNA Concentration Range (ng/µL)	RNA Integrity Number
30 min Control (IAA)	109 – 146	9.4 – 9.9
30 min IAA	214 – 287	9.7 – 9.9
4 h Control (IAA)	66 – 161	8.2 – 9.7
4 h IAA	190 – 214	9.7 – 9.8
30 min Control (BAA)	215 – 230	9.9 – 10.0
30 min BAA	280 – 343	9.9 – 10.0
4 h Control (BAA)	43 – 68	9.4 – 9.6
4 h BAA	28 – 259	8.4 – 9.9
30 min Control (CAA)	117 – 185	9.9 – 10.0
30 min CAA	138 – 235	9.8 – 10.0
4 h Control (CAA)	120 – 206	9.8 – 10.0
4 h CAA	94 – 232	9.8 – 10.0

Table 3.4. SABiosciences Human Oxidative Stress and Antioxidant Defense RT^2 Profiler PCR Array PAHS-065A		
Description	**Gene Symbol**	**Functional Gene Group(s)**
Albumin	ALB	Antioxidant
Arachidonate 12-lipoxygenase	ALOX12	Superoxide Metabolism
Angiopoietin-like 7	ANGPT7L	Oxidative Stress Repsonsive
Aldehyde oxidase 1	AOX1	ROS Metabolism
Apolipoprotein E	APOE	Antioxidant, Oxidative Stress Responsive
ATX1 antioxidant protein 1 homolog (yeast)	ATOX1	Oxidative Stress Responsive
BCL2/adenovirus E1B 19kDa interacting protein 3	BNIP3	ROS Metabolism
Catalase	CAT	Peroxidase, Oxidative Stress Responsive
Chemokine (C-C motif) ligand 5	CCL5	Oxidative Stress Responsive
Copper chaperone for superoxide dismutase	CCS	Superoxide Metabolism
Cold shock domain containing E1, RNA-binding	CSDE1	Peroxidase, Oxidative Stress Responsive
Cytochrome b-245, alpha polypeptide	CYBA	Superoxide Metabolism
Cytoglobin	CYGB	Peroxidase, Oxidative Stress Responsive
Diacylglycerol kinase, kappa	DGKK	Oxidative Stress Responsive
24-dehydrocholesterol reductase	DHCR24	Oxidative Stress Responsive
Dual oxidase 1	DUOX1	Peroxidase, Superoxide Metabolism, Oxidative Stress Responsive
Dual oxidase 2	DUOX2	Peroxidase, Oxidative Stress Responsive
Dual specificity phosphatase 1	DUSP1	Oxidative Stress Repsonsive
Epoxide hydrolase 2, cytoplasmic	EPHX2	ROS Metabolism
Eosinophil peroxidase	EPX	Peroxidase, Oxidative Stress Responsive
Forkhead box M1	FOXM1	Oxidative Stress Responsive
General transcription factor II,i	GTF2I	Superoxide Metabolism
Glutaredoxin 2	GLRX2	Oxidative Stress Repsonsive
G protein-coupled receptor 156	GPR156	Peroxidase, Oxidative Stress Responsive
Glutathione peroxidase 1	GPX1	Glutathione Peroxidase, Oxidative Stress Responsive

Table 3.4. (cont.)		
Glutathione peroxidease 2 (gastrointestinal)	GPX2	Glutathione Peroxidase, Oxidative Stress Responsive
Glutathione peroxidase 3 (plasma)	GPX3	Glutathione Peroxidase, Oxidative Stress Responsive
Glutathione peroxidase 4 (phospholipid hydroperoxidase)	GPX4	Glutathione Peroxidase, Oxidative Stress Responsive
Glutathione peroxidase 5 (epididymal androgen-related protein)	GPX5	Glutathione Peroxidase, Oxidative Stress Responsive
Glutathione peroxidase 6 (olfactory)	GPX6	Glutathione Peroxidase, Oxidative Stress Responsive
Glutathione peroxidase 7	GPX7	Glutathione Peroxidase, Oxidative Stress Responsive
Glutathione reductase	GSR	Antioxidant
Glutathione synthetase	GSS	Oxidative Stress Responsive
Glutathione transferase zeta 1 (maleylacetocetate isomerase)	GSTZ1	Glutatathione Peroxidase
Keratin 1	KRT1	Oxidative Stress Responsive
Lactoperoxidase	LPO	Peroxidase, Oxidative Stress Responsive
Mannose-binding lectin (protein C) 2, soluble (opsonic defect)	MBL2	Oxidative Stress Responsive
Microsomal glutathione S-transferase 3	MGST3	Peroxidase
Myeloperoxidase	MPO	Peroxidase, Oxidative Stress Responsive
MpV17 mitochondrial inner membrane protein	MPV17	ROS Metabolism
Methionine sulfoxide reductase A	MSRA	Oxidative Stress Responsive
Metallothionein 3	MT3	Antioxidant, Superoxide Metabolism
Metallothionein-like 5, testis-specific (tesmin)	MTL5	Oxidative Stress Repsonsive
Neutrophil cytosolic factor 1, (chronic granulomatous disease, autosomal 1)	NCF1	Superoxide Metabolism
Neutrophil cytosolic factor 2, (65kDa, chronic granulomatous disease, autosomal 2)	NCF2	Superoxide Metabolism
Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	NME5	Oxidative Stress Responsive
Nitric oxide synthase 2A, inducible	NOS2A	Superoxide Metabolism

Table 3.4. (cont.)		
NADPH oxidase, EF-hand calcium binding domain 5	NOX5	Superoxide Metabolism
Nudix (nucleoside diphosphate linked moiety X)-type motif 1	NUDT1	Oxidative Stress Responsive
Oxidation resistance 1	OXR1	Oxidative Stress Responsive
Oxidative-stress responsive 1	OXSR1	Oxidative Stress Responsive
PDZ and LIM domain 1 (elfin)	PDLIM1	Oxidative Stress Responsive
Phosphoinositide-binding protein PIP3-E	PIP3-E	Peroxidase, Oxidative Stress Responsive
Polynucleotide kinase 3'-phosphatase	PNKP	Oxidative Stress Responsive
Peroxiredoxin 1	PRDX1	Peroxiredoxin
Peroxiredoxin 2	PRDX2	Peroxiredoxin, Oxidative Stress Responsive
Peroxiredoxin 3	PRDX3	Peroxiredoxin
Peroxiredoxin 4	PRDX4	Peroxiredoxin
Peroxiredoxin 5	PRDX5	Peroxiredoxin, Oxidative Stress Responsive
Peroxiredoxin 6	PRDX6	Peroxiredoxin, Oxidative Stress Responsive
Phophatidylinositol, 3,4,5-triphosphate-dependent RAC exchanger 1	PREX1	Superoxide Metabolism
Proteoglycan 3	PRG3	Superoxide Metabolism
Prion protein (p27-30) (Creeutzfeldt-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia)	PRNP	Oxidative Stress Responsive
Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	PTGS1	Peroxidase
Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	Peroxidase
Peroxidasin homolog (Drosophila)	PXDN	Peroxidase
Peroxidasin homolog (Drosophila)-like	PXDNL	Peroxidase
Ring finger protein 7	RNF7	Oxidative Stress Responsive
Scavenger receptor class A, member 3	SCARA3	Oxidative Stress Responsive
Selenoprotein S	SELS	Antioxidant, Oxidative Stress Responsive
Selenoprotein P, plasma, 1	SEPP1	Oxidative Stress Responsive

Table 3.4. (cont.)		
Surfactant protein D	SFTPD	ROS Metabolism
Serum/glucocorticoid regulated kinase 2	SGK2	Oxidative Stress Responsive
Sirtuin (silent mating type information regulation 2 homolog) 2 (S. cerevisiae)	SIRT2	Oxidative Stress Responsive
Superoxide dismutase 1, soluble (amytrophic lateral sclerosis 1 (adult))	SOD1	Antioxidant, Superoxide Dismutase, Oxidative Stress Responsive
Superoxide dismutase 2, mitochondrial	SOD2	Superoxide Dismutase, Oxidative Stress Responsive
Superoxide dismutase 3, extracellular	SOD3	Antioxidant, Superoxide Dismutase
Sulfiredoxin 1 homolog (S. cerevisiae)	SRXN1	Antioxidant, Oxidative Stress Responsive
Serine/threonine kinase 25 (STE20 homolog, yeast)	STK25	Oxidative Stress Responsive
Thyroid peroxidase	TPO	Peroxidase, Oxidative Stress Responsive
Titin	TTN	Peroxidase, Oxidative Stress Responsive
Thioredoxin domain containing 2 (spermatozoa)	TXNDC2	Antioxidant
Thioredoxin reductase 1	TXNRD1	Antioxidant
Thioredoxin reductase 2	TXNRD2	Antioxidant, Oxidative Stress Responsive

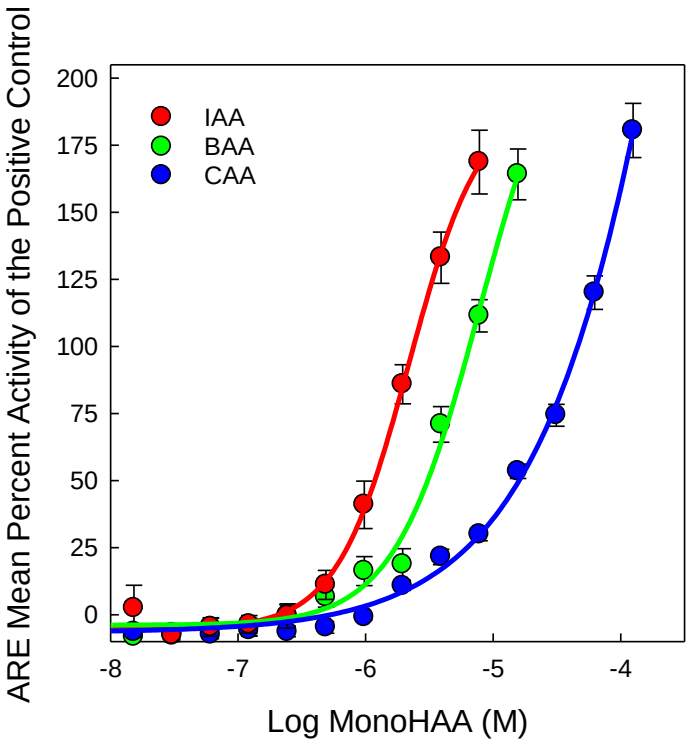

Figure 3.1. Concentration-response curves for IAA, BAA and CAA for the induced expression of the ARE-controlled β-lactamase reporter gene in ARE-bla HepG2 cells. The data are presented as the mean percent activity (±SE) of the positive control (23 μM β-napthoflavone).

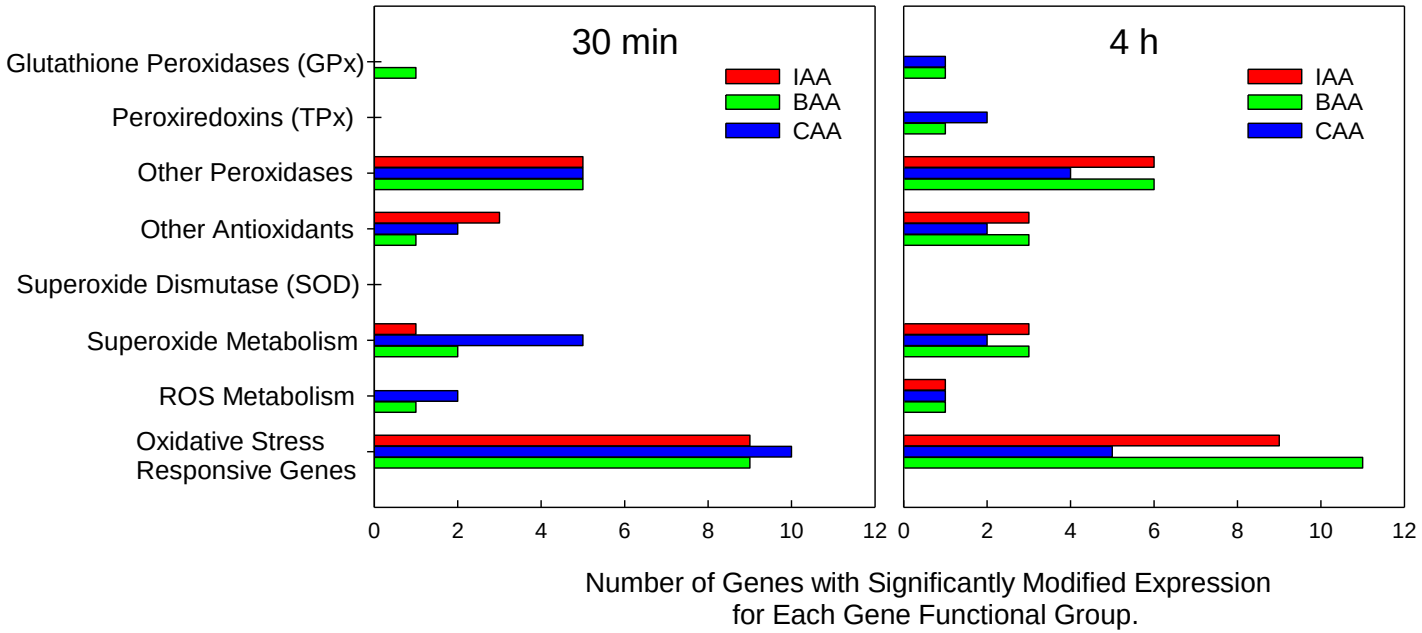

Figure 3.2. The number of ROS-responding genes listed by functional groups that exhibited altered expression after 30 min or 4 h exposure to 22 µM IAA, 57 µM BAA, or 3.4 mM CAA. Some genes were counted more than once because of their involvement in multiple pathways.

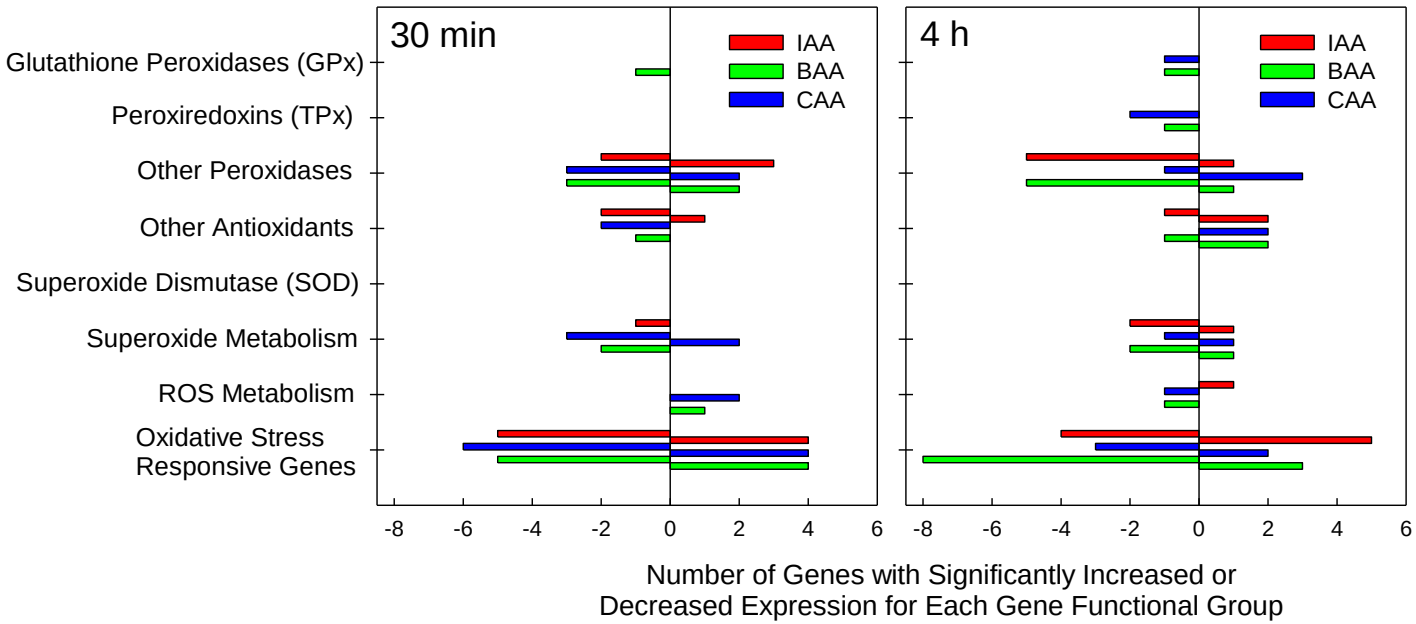

Figure 3.3. The number of oxidative stress responsive genes listed by functional groups illustrating upregulation or downregulation after 30 min or 4 h exposure to 22 µM IAA, 57 µM BAA, or 3.4 mM CAA. Some genes were counted more than once because of their involvement in multiple pathways.

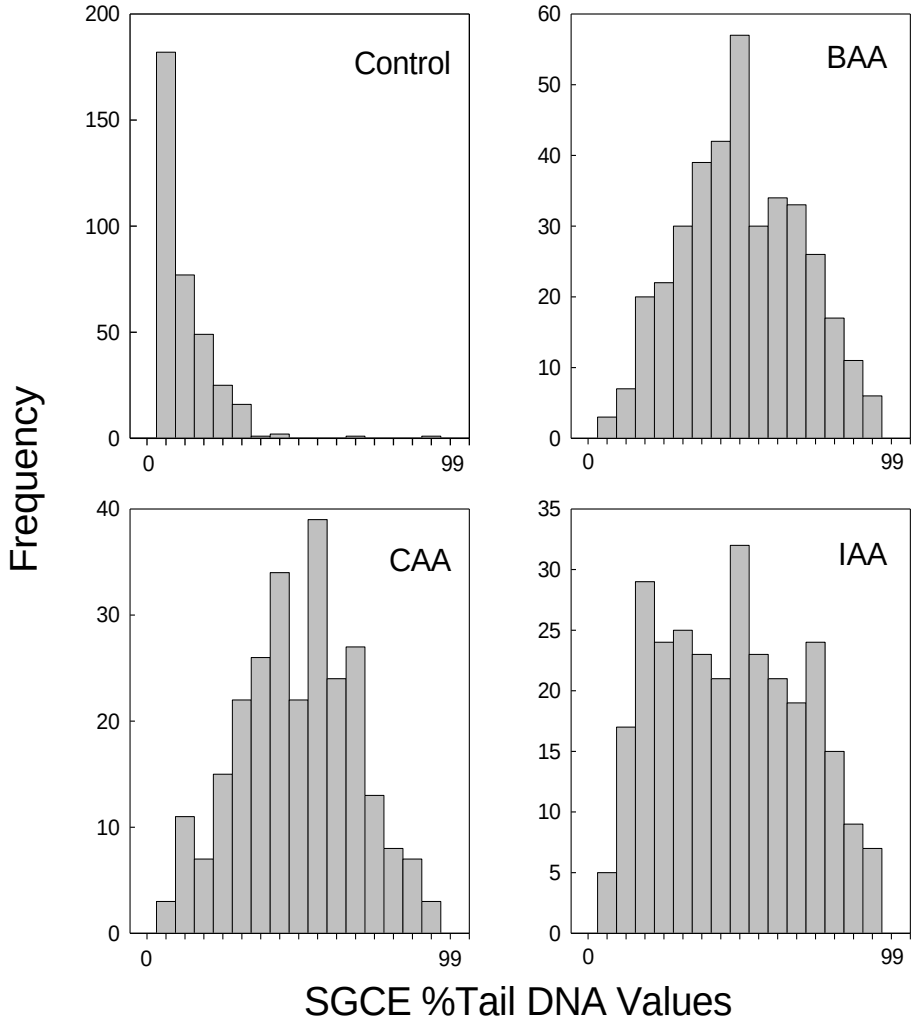

Figure 3.4. Histograms of the distribution of SCGE genomic DNA damage in human FHs cells for the negative control, and for cells that expressed an average value of 50 %Tail DNA for BAA, CAA and IAA-induced genotoxic damage.

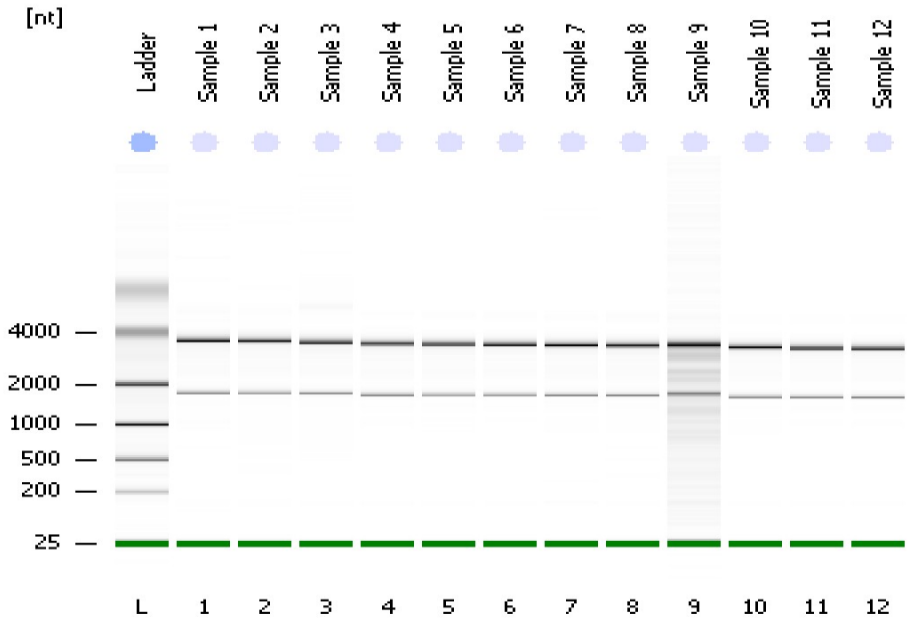

Figure 3.5. Electrophoresis of RNA samples isolated from control and FHs cells treated with IAA.

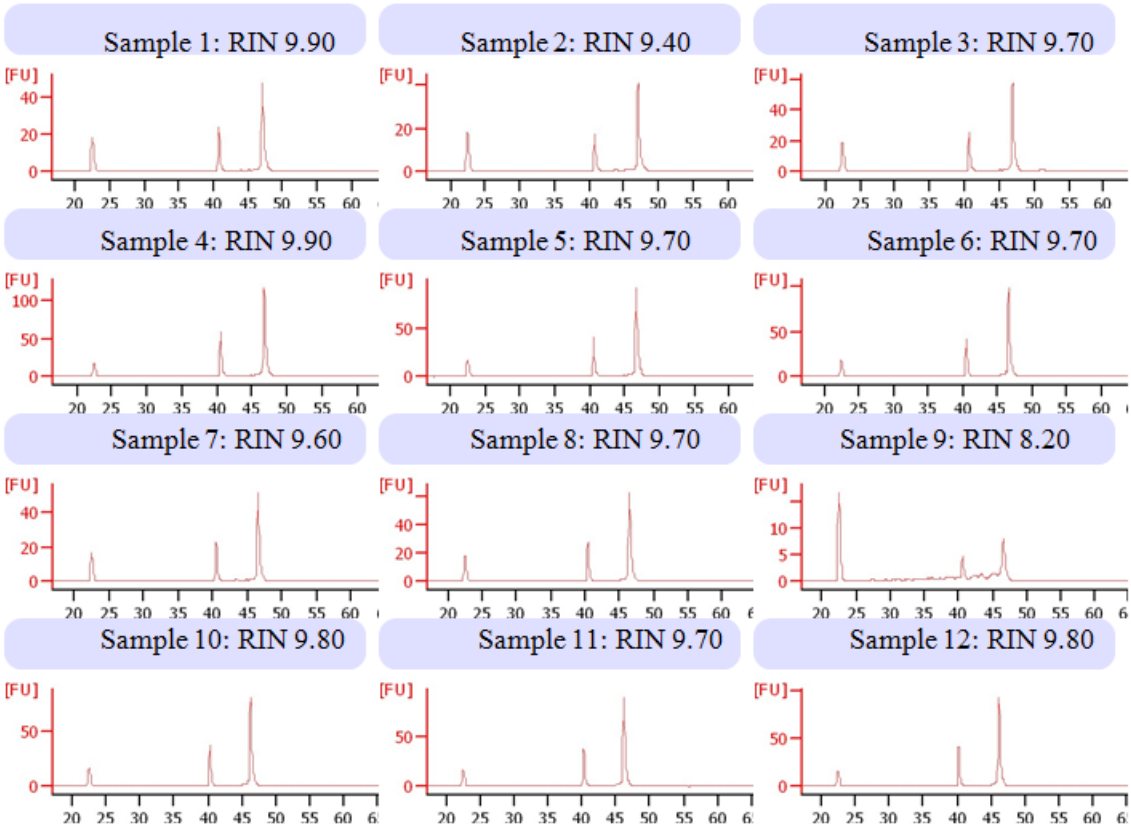

Figure 3.6. Capillary electrophoresis of RNA isolated from concurrent negative controls and IAA-treated human FHs cells. Note RIN values for RNA quality exceed the minimum value of 7.0.

CHAPTER 4: CALCIUM AND REACTIVE OXYGEN SPECIES IN DBP INDUCED GENOTOXICITY: IS THERE A CUMULATIVE EFFECT?

Introduction

Disinfection of drinking water greatly improved public health by reducing diseases transmitted through drinking water [1]. Chemical disinfectants, such as chlorine, chloramines, or ozone kill pathogenic microorganisms otherwise present in drinking water, but also react with organic and inorganic materials forming toxic disinfection byproducts (DBPs) as an unintended consequence. Since they were first discovered in 1974 [2, 3] over 600 individual DBPs have been discovered in disinfected water, where they are generated at relatively low (ranging from low μg/L to ng/L) concentrations [4].

Long term exposure to DBPs was associated with small but significant increased risks for colorectal [5] and bladder cancers [6]; exposure to DBPs prior to and during gestation was also associated with increased risk for adverse pregnancy outcomes [7]. Individual DBPs were genotoxic, mutagenic, carcinogenic, and teratogenic (reviewed in [8]). However, the toxicity of these DBPs individually cannot account for the increased risk of cancer measured in the epidemiology because they did not cause bladder or colon cancers and the carcinogenic concentrations in laboratory animals would not be achieved by drinking, bathing, and/or swimming in disinfected water [9].

Although the link between DBP exposure to cancer and adverse pregnancy outcomes are not known, oxidative stress and genotoxicity were suggested as possible mechanisms [10]. In a systematic evaluation of DBP toxicity in a Chinese hamster ovary (CHO) model cell line monohalogenated haloacetic acids (monoHAAs) [11], acetonitriles (monoHANs) [12], and acetamides (monoHAMs) [13] were among the most genotoxic. Members of each of these

classes generated biomarkers of oxidative stress [14-16]. Buffering intracellular calcium (Ca^{2+}) with EGTA-AM [15] or BAPTA-AM [17] reduced toxicity generated by iodoacetamide (IAM) and iodoacetate (IOA), respectively, and also reduced the generation of reactive oxygen species (ROS) in iodoacetate treated neurons [18]. While IOA (and presumably iodoacetic acid) and IAM shared increased intracellular Ca^{2+} and the generation of ROS in their respective toxic cascades, different mechanisms have been proposed for initiating their toxicities. IAA is a potent inhibitor of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and its toxicity is thought to be derived from this inhibition [19]; the ability to inhibit GAPDH strongly correlated with multiple toxicological endpoints for each of the monoHAAs, which suggested inhibition of GAPDH was shared as a mechanism among these compounds [20]. IAM is a weaker inhibitor of GAPDH, but reacts more readily with glutathione (GSH) and depletion of this important intracellular antioxidant and electrophile scavenging tripeptide is likely initiating the toxic cascade associated with this compound [19]. The monoHANs are structurally similar to both the monoHAAs and monoHAMS and reacted with GSH [21]. ^{14}C labeled chloroacetonitrile (CAN) was detected in DNA; however, this reactivity was dependent on GSH depletion [22]. Induction of oxidative stress was an important initiator of toxicity for CAN under physiological GSH concentrations [14, 23].

The purpose of this research was to first investigate the roles ROS and intracellular Ca^{2+} play in the genotoxicity induced by the monoHAAs. We then extended this research to include bromoacetamide (BAM), and bromoacetonitrile (BAN) so as to determine if multiple chemical classes generated in disinfected waters might converge at these steps in a cumulative toxic cascade.

Evaluating the toxicity of DBP mixtures will provide needed information linking DBP exposures to the adverse health outcomes measured in epidemiologic studies. In a series of studies Dawson et al evaluated interactions of binary mixtures of S_N2 reactive electrophiles using a cytotoxicity endpoint [21, 24, 25]. Within the monoHANs, iodoacetonitrile, BAN, and CAN showed additive toxicity, which, the authors suggested, indicated a single shared mechanism [21]. Ethyl bromoacetate, and ethyl iodoacetate also showed additive toxicity [24] as did the mixture of these compounds with the monoHANs [25], suggesting that members of these two chemical classes interact to generate toxicity. To gain a better understanding of the cumulative effect of multiple DBP classes on genotoxicity we used SCGE to evaluate DNA damage induced by defined component mixtures of bromoacetic acid (BAA), BAN, and BAM, three structurally related, S_N2 reactive, electophilic chemicals that generate oxidative stress through different mechanisms.

Materials and Methods

Chemicals and Reagents

General reagents were purchased from Fisher Scientific Co. (Itasca, IL) or Aldrich Chemical Co. (St. Louis, MO). Bromoacetic acid and chloroacetic acid were purchased from Fluka Chemical Co. (Buchs, Switzerland). Iodoacetic acid, bromoacetonitrile (97%,), butylated hydroxianisole (BHA), N-acetyl L-cysteine (NAC) and 1,2- bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)(BAPTA-AM) were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture medium (Hamm's F12) and fetal bovine serum (FBS) were purchased from Fisher Scientific Co. Stock solutions of IAA, BAA, CAA, BAN,

BAM, BHA (1 M) and) BAPTA-AM (50 mM) were prepared in DMSO and stored in sterile glass vials at -22 °C. Stock solutions were diluted in F12 medium immediately prior to use.

Cell Culture

Chinese hamster ovary (CHO) cell line AS52 were maintained in Hamm's F12 medium supplemented with 5% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 atmosphere. Cells were grown on 100 mm glass tissue culture plates.

Single Cell Gel Electrophoresis

Single cell gel electrophoresis was used to measure genomic DNA damage in response to various agents. Minor modifications to the detailed procedure previously published [26] were used. Briefly, 4×10^5 CHO cells in Hamm's F12 medium supplemented with 5 % FBS were added to each well of a 96 well microtiter plate. The cells were incubated overnight in a humidified 5% CO_2 atmosphere at 37 °C. Immediately prior to DBP exposure, the medium was aspirated and cells were washed twice with 100 µL of Hank's balanced salt solution (HBSS). Cells were treated with DBP in 25 µL of F12 medium (without serum) for 4 h at 37 °C and 5% CO_2. Wells were covered with sterile Alumnaseal ™ during treatment. After the exposure period medium was aspirated, cells were washed twice with HBSS and harvested with 50 µL trypsin (0.005% in HBSS). 70 µL of F12 + 5% FBS was added to inactivate the trypsin. After a 10 µL aliquot of cell suspension was reserved for acute cytotoxicity evaluation, the remainder was mixed with 120 µL 1% low melting point agarose (LMA) prepared in phosphate buffered saline. 90 µL of the agarose cell suspension was pipetted onto a microscope slide previous covered with 1% normal melting point agarose (prepared in deionized water); a cover slip was

added to distribute the agarose suspension. An additional layer of LMA (0.5%) was added after the initial layer solidified. The gels were submerged overnight in lysing solution (2.5 M NaCl, 100 mM Na2 EDTA, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100, and 10% DMSO) at 4 °C. The DNA was denatured in alkaline electrophoresis buffer (300 mM NaOH, 1mM EDTA) for 20 minutes and then electrophoresed at 72 V/cm (25 V, 300 mA) for 40 min at 4 °C. After electrophoresis the slides were removed from electrophoresis buffer and neutralized with 700 mM Tris buffer pH 7.5. The slides were rinsed in cold deionized water, dehydrated in methanol for 20 min at 4 °C and then dried for 10 min in a 50 °C oven. The gels were rehydrated in 4 °C deionized water for 30 min and stained with ethidium bromide (60 μg/mL) for 5 min. DNA damage was quantified as mean % tail DNA under a Zeiss fluorescence microscope using the Comet IV imaging system (Perspective Instruments, Suffolk UK).

Acute toxicity for treated and control cells was evaluated with the Trypan blue (0.05% in phosphate buffered saline) vital dye exclusion assay. Cell viability was ≥ 90% for all experiments (data not shown).

The Effect of BHA on MonoHAA Induced DNA Damage

To measure the effect of the antioxidant butylated hydroxianisole (BHA), cells were exposed to IAA (15 μM), BAA (30 μM) or CAA (5 mM), concentrations chosen to induce a similar amount of genomic DNA damage. In addition to mono-HAA some of the cells were co-treated with BHA (100, 250, or 500 μM). DNA damage was measured using the SCGE assay described previously, and a concentration response relationship for the BHA mediated effect on monoHAA induced DNA damage was evaluated by comparing positive control cells (monoHAA alone) to monoHAA + BHA co-treated cells. Positive control % tail DNA values were set to

100% and the monoHAA + BHA data were normalized to, and reported as a percentage of their respective positive controls.

The Effect of BAPTA-AM on DBP Induced DNA Damage

To measure the effect of the specific intracellular calcium chelator, BAPTA-AM on genotoxicity of individual DBPs, CHO cells were exposed to IAA (25 μM), BAA (75 μM), CAA (5 mM), BAN (60 μM) or BAM (60 μM), the genomic DNA damage induced by DBP alone served as the positive control value (100%) for each compound. BAPTA-AM (100 μM or 200 μM) was co-treated with each of the monoHAAs, BAN, or BAM to measure the effect of intracellular Ca^{2+} chelation on genomic DNA damage induced by each of the DBPs. The mean SCGE % tail DNA was calculated for each BAPTA-AM concentration. The positive control values were set to 100%; data for DBP + BAPTA-AM were reported as the % of their respective positive control. Ethylmethanesulfonate (EMS) (3.8 mM) and hydrogen peroxide (H_2O_2) (0.003% v/v) served as additional non-specific DNA damage controls.

The Effect of NAC on Monobrominated DBP Induced DNA Damage

To investigate a possible role of GSH depletion in the induction of genomic DNA damage by BAN, BAM, and BAA, the protective effect of the GSH precursor, NAC, was measured. CHO cells were treated with BAA (250 μM), BAN (60 μM) or BAM (60 μM) in the absence and presence of NAC (100 μM, 250 μM, or 500 μM). DNA damage was measured using the SCGE assay described previously. CHO cells treated with DBP alone served as a positive control; positive control SCGE % tail DNA values were set to 100% and the DBP + NAC treated cell % tail DNA data were normalized to their respective positive controls.

Genotoxicity of Monobrominated DBP Mixtures

To investigate the interaction of binary mixtures of BAA, BAN, and BAM, CHO cells were treated with a low concentration (30 µM BAN and BAM, or 125 µM BAA) a high concentration (60 µM BAN and BAM, or 250 µM BAA), and a mixture of the low concentrations. Genomic DNA damage was measured using SCGE methods described above. DNA damage for the mixtures was compared to DNA damage induced by individual DBPS.

Statistical Analyses

For DNA damage the unit of measure for each microgel was the average % tail DNA, which is the amount of DNA that migrated into the gel from the nucleus. The mean % tail DNA values were calculated for the microgels in a treatment group and were analyzed with an ANOVA statistical test. If a significant F value of $P < 0.05$ was obtained, a Holm-Sidak pairwise comparison versus the control group analysis was conducted (power ≥ 0.8 at $\alpha = 0.05$).

Results and Discussion

Calcium and Reactive Oxygen Species in MonoHAA Induced Genotoxicity

The monoHAAs were genotoxic in CHO cells [26, 27], but did not induce damage in acellular DNA [20]. IAA inhibited the glycolytic enzyme GAPDH and generated a neurotoxic cascade that included disruption of intracellular calcium homeostasis (increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)) and the generation of ROS [18]. ROS can damage DNA directly or indirectly by generating byproducts of lipid peroxidation, such as malondialdehyde and 4-hydroxynonenal, which are genotoxic [28]. Therefore, generation of ROS subsequent to IAA exposure was investigated as a possible mechanism for genotoxicity; the antioxidants BHA and

catalase reduced genomic DNA damage in CHO cells exposed to IAA [29]. Each of the monoHAAs inhibited GAPDH activity [20] and generated biomarkers of oxidative stress [16], indicating they shared a similar mode of toxic action. Here we investigated the involvement of ROS and Ca^{2+} in monoHAA induced genomic DNA damage.

As previously reported [26, 27], each of the monoHAAs induced genomic DNA damage in CHO AS52 cells (Figure 4.1 and 4.2). Concentrations of monoHAAs that generated approximately similar SCGE % tail DNA values were chosen for further analysis. IAA (15 μM), BAA (30 μM), and CAA (5 mM) alone served as positive control values. Co-treating the cells with BHA 250 μM and 500 μM BHA significantly ($P \leq 0.001$ for IAA vs. IAA + 250 μM, IAA vs. IAA + 500 μm BHA, BAA vs. BAA + 500 μM BHA and CAA vs. CAA + 500 μM BHA; $P \leq 0.020$ for BAA vs. BAA + 250 μM BHA; and $P \leq 0.008$ for CAA vs. CAA 250 μM BHA) reduced the amount of damage that accumulated in the nuclei during monoHAA exposures (Figure 4.1). A BHA concentration dependent reduction of monoHAA induced genomic DNA damage was observed (Figure 4.1), however, at the highest BHA concentration tested (500 μM), a significant amount of DNA (62.8%, 42.9 %, and 81.5% for IAA, BAA, and CAA respectively) migrated into the tail under the electrophoretic force. The antioxidants vitamin E (α-tocopherol) and nordihydroguaiaretic acid (NDGA) offered more substantial protection against iodoacetate induced neurotoxicity [18], but in general our data supported the hypothesis that monoHAA derived genotoxicity is dependent on the generation of ROS.

Co-treating the cells with the calcium specific intracellular chelator, BAPTA-AM, also significantly reduced the genotoxicity induced by IAA (30 μM), BAA (75 μM), and CAA (5 mM) (Figure 4.2). BAPTA-AM at 100 μM produced a significant ($P \leq 0.001$) reduction of % tail DNA compared to positive controls for IAA and CAA. Buffering $[Ca^{2+}]_i$ with 200 μM BAPTA-

AM reduced DNA damage induced by each of the monoHAAs by > 75% (Figure 4.2). To assure the effect of BAPTA-AM on monoHAA-induced genotoxicity was not artifactual, its effect on two additional DNA damaging agents was evaluated. BAPTA-AM (200 µM) provided a small (<10%) but significant ($P \leq 0.001$) protective effect when compared to EMS alone (Figure 4.3). BAPTA-AM (200 µM) reduced DNA damage induced by H_2O_2 by ~ 20% ($P \leq 0.001$) (Figure 4.3). Compared to the EMS and H_2O_2 controls BAPTA-AM had a much greater effect on monoHAA induced genotoxicity (Figure 4.2) these data eliminated concerns of a non-specific protective effect.

The monoHAA + BAPTA-AM data indicate that elevated $[Ca^{2+}]_i$ played a critical role in monoHAA induced genotoxicity. Buffering cytosolic Ca^{2+} in hippocampal neurons effectively reduced ROS generated after IAA exposure [18]. Taken with the ability of antioxidants to reduce monoHAA induced genomic DNA damage shown here (Figure 1.1) and previously [29], these data suggest that elevated $[Ca^{2+}]_i$ is involved in the generation of ROS that damage DNA.

Glycolysis is closely linked with maintaining Ca^{2+} homeostasis, especially within the endoplasmic/sarcoplasmic reticulum (ER/SR). Glycolytic enzymes were found associated on the SR membrane where they could produce a local ATP concentration used to fuel the SR/ER Ca^{2+} ATPase (SERCA), which pumps Ca^{2+} down its chemical gradient into the ER/SR; IAA inhibited SERCA activity indirectly as exogenous ATP restored Ca^{2+} influx into the SR [30]. Glycolytic inhibitors, including IAA depleted intracellular Ca^{2+} stores in hippocampal neurons much more efficiently than mitochondrial inhibitors further implicated local ATP production maintaining Ca^{2+} homeostasis [31]. Toxicological studies using the irreversible SERCA inhibitor thapsigargin showed that disrupting SERCA function is sufficient to generate ROS, peroxidation of the ER membrane, and ER stress [32, 33].

Calcium and GSH in BAN, BAM, and BAA Induced Toxicity

Disruption of Ca^{2+} homeostasis was also implicated in IAM toxicity, as EGTA-AM, an intracellular Ca^{2+} chelator, reduced cytotoxicity induced by this chemical [15]. The cytotoxic mode of action proposed for IAM includes depletion of GSH, protein aggregation via intermolecular disulfide bonding, disruption of ER Ca^{2+} homeostasis and generation of ROS [34]. CAN depleted GSH and induced oxidative stress in mouse embryos *in vivo* [23]. Because monoHAAs, monoHANs, and monoHAMs are all present in disinfected water, it is important to investigate how these toxicants might interact. We wanted to determine if increased $[Ca^{2+}]_i$ was contributing to genotoxicity induced by the monoHANs and monoHAMs. It was determined through investigating binary mixtures that each of the monoHANs act through a common mechanism [21], thus we selected the brominated species, BAN and BAM, as models for further investigation and compared these with BAA.

As previously demonstrated [12, 13], both BAN (60 μM), and BAM (60 μM) generated significant genomic DNA damage in CHO cells (Figure 4.4). BAPTA-AM reduced BAN and BAM genotoxicity in a concentration dependent manner (Figure 4.4). The highest BAPTA-AM concentration (200 μM) significantly ($P \leq 0.001$) reduced BAN and BAM induced DNA damage by 63.9% and 73.6% relative to the positive controls, respectively. BAPTA-AM's effect on BAN and BAM induced genotoxicity was comparable to the 76.0% reduction of BAA induced DNA damage (Figure 4.4).

Based on the data presented in Figure 4.4, each of BAN, BAM, and BAA required increased $[Ca^{2+}]_i$ to reach their maximum genotoxic potency. However, different mechanisms of intracellular Ca^{2+} homeostasis disruption have been proposed, with GSH depletion for BAN and BAM, and GAPDH inhibition being the initiating event proposed for BAA. To further

investigate the role GSH plays in the toxicity of these compounds exogenous NAC, a GSH precursor, was co-treated with BAN, BAM, and BAA. Genotoxic concentrations of each compound (60 μM BAN and BAM, and 250 μM BAA) served as positive controls; NAC was supplemented at 100 μM, 250 μM, and 500 μM and % tail DNA values for co-treated cells are reported as % of their respective positive controls (Figure 4.5). For BAN and BAM a clear NAC concentration dependent reduction of DNA damage is evident (Figure 4.5); significant ($P \leq$ 0.001) reductions of DNA damage occurred at 100 μM NAC and 250 μM NAC for BAN and BAM, respectively. NAC did not protect the CHO cells from BAA induced DNA damage, although there appeared to be an indication of some protective effect emerging at the highest NAC concentration (Figure 4.5). NAC can directly scavenge electrophiles or provide an extracellular source of cysteine to enhance production of GSH [35]. It cannot be determined form these experiments if NAC or GSH are directly interacting with BAN and BAM or neutralizing ROS generated from some other pathway, but, in either case, the differential effect of NAC on BAN and BAM, vs. BAA induced genotoxicity suggest these electrophilic DBPs target different intracellular nucleophiles.

Cumulative Toxicity Among Monobrominated-DBP Binary Mixtures

Because they have different cellular targets, it is unclear how these DBPs would interact in mixtures, while the proposed initiating events are different; they all potentially converge at disruption of ER Ca^{2+} homeostasis [31, 34]. To investigate how these chemicals interact we prepared binary mixtures and measured genotoxicity with SCGE as an endpoint. Mixtures included sham mixtures (two “low” concentrations of the same chemical to generate a single “high” concentration) or mixtures of two chemicals at the defined “low” concentrations. For

BAN, and BAM the “low” and “high” concentrations were 30 μM and 60 μM, respectively; for BAA the “low” and “high” concentrations were 125 μM, and 250 μM.

Exposures to 30 μM BAN (8.9 % tail DNA) was less genotoxic than 30 μM BAM (30.4 % tail DNA); the mixture of 30 μM BAN plus 30 μM BAM was less genotoxic (60.1 % tail DNA) than 60 μM of either BAN or BAM (71.3, and 76.4 % tail DNA, respectively) but 153.3% of the sum of the BAN + BAM low concentrations (39.2 % tail DNA) (Figure 4.6).

The mean % tail DNA value (29.0%) resulting from binary mixtures of 30 μM BAN and 125 μM BAA was similar to the sum of the two individual % tail DNA values generated from the low concentrations of BAN and BAA (31.7 %). The mixture was not statistically different from 125 μM BAA (21.3 % tail DNA; $P \leq 0.078$) or 250 μM BAA (34.1 % tail DNA; $P \leq 0.273$), but greater than 30 μM BAN (10.4 % tail DNA; $P \leq 0.001$) and less than 60 μM BAN (70.9 % tail DNA; $P \leq 0.001$) (Figure 4.7).

The mean % tail DNA resulting from binary mixtures of 30 μM BAM and 125 μM BAA (60.9 % tail DNA) was similar to the sum (63.9% tail DNA) of 30 μM BAM (34.5 % Tail DNA) and 125 μM BAA (29.3 % tail DNA). The mixture was more genotoxic than either of the individual BAA concentrations and 30 μM BAM, but generated less DNA damage than 60 μM BAM (78.9 % tail DNA) ($P \leq 0.001$ in each case) (Figure 4.8).

Evaluating combined toxicity was used to determine if S_N2 reactive soft electrophilic compounds shared a similar toxic mechanism; where it was suggested that purely additive toxicities indicated a single shared mechanism [21, 24, 25]. Of the binary mixtures investigated in this study BAM + BAA and BAN + BAA seemed to produce an effect equal to the sum of the individual exposures. Although these data agreed with a previous report of additivity among monoHANs and ethyl halogenated acetates (chemicals structurally related to the monoHAAs),

this phenomenon was somewhat peculiar, as the two compounds in each mixture have different proposed modes of action, and BAN and BAM, both of which deplete cellular GSH, had a greater than additive genotoxic effect. While the proposed cellular targets are different (GAPDH for BAA, and GSH for BAN and BAM) each of the compounds are structurally similar. Alkyl halide functional groups (C-X, where X is chlorine, bromine or iodine), due to the electron withdrawing effect of the halogen and the subsequent partial positive charge (lack of electron density) on the carbon atom are S_N2 reactive electrophiles. Electrophilic compounds pose a threat to living systems. By reacting with biological nucleophiles, i.e. nitrogen or oxygen atoms in DNA, or nitrogen, oxygen, and sulfur atoms in proteins, electrophiles can disrupt the function of proteins or damage DNA leading to mutations and or cell death. The hard/soft designation assigned acids and bases can be used to predict reactivity of electro/nucleophile pairs; in general, hard-hard or soft-soft pairs are more reactive than a hard-soft combination [36]. Sulfur containing thiols (R-SH) or thiolate anion ($R\text{-}S^-$), being the softest of the biological nucleophiles, were most reactive with soft electrophiles [37]. The sulfhydryl (SH) groups in cysteine residues play a critical role in sensing and eliminating electrophilic and oxidative stress in living systems [38]. Glutathione (GSH) and thioredoxin utilize cysteine residues to bind electrophilic molecules in an attempt to detoxify the cellular environment and prevent accumulation of ROS or aggregation of proteins during oxidative stress [38]. Additionally the transcription factor Nrf2 is dependent on cysteine residues in its regulator Keap1 for its activation [39]. Electrophilic or oxidative stress causes intermolecular S-S bridge changing the conformation of Keap1 dimers [40, 41], which in turn releases Nrf2 allowing it to translocate to the nucleus where it binds to antioxidant response elements (ARE), cis acting elements in DNA that regulate the production of a host of antioxidant enzymes including those that synthesize GSH [42].

Because GSH plays a critical role in maintaining redox balance, compounds that deplete GSH lead to oxidative stress, protein aggregation via intermolecular S-S bonding, and ultimately toxicity. Included among the proteins that aggregate during oxidative stress is GAPDH [43]; oxidation or poly (ADP-ribosyl)ation of GAPDH's active site cysteine by poly (ADP-ribose) polymerase (PARP), which is activated by DNA strand breaks, also inactivate the glycolytic function of the enzyme [44]. Embryonic GAPDH was also inhibited by oxidative stress and played an important role in hydroxyurea induced teratogenesis [45].

Alternatively inhibition of GAPDH causes a rapid depletion of cellular ATP [46] which is required to generate GSH [47]. Disruption of glycolysis also inhibited SERCA activity [30, 31] which led to increased $[Ca^{2+}]_i$ and generated ROS and lipid peroxidation [32, 33] which in turn depleted GSH [47]. So, while the exact same ROS-inducing mechanisms might not exist among HANs, HAMs, and HAAs, they are each soft electrophiles that target sulfhydryl groups, and disruption of glycolysis and depletion of cellular GSH feedback upon each other to disrupt Ca^{2+} homeostasis and generate oxidative stress. These findings provide an important step in considering the cumulative effect of multiple DBPs, whereas no individual DBP is likely to be generated at a toxic concentration, many soft electrophilic compounds are generated during the disinfection process and could act in concert to disrupt intracellular Ca^{2+} homeostasis and generate oxidative stress, which might then lead to cancer and adverse pregnancy outcomes.

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Figures

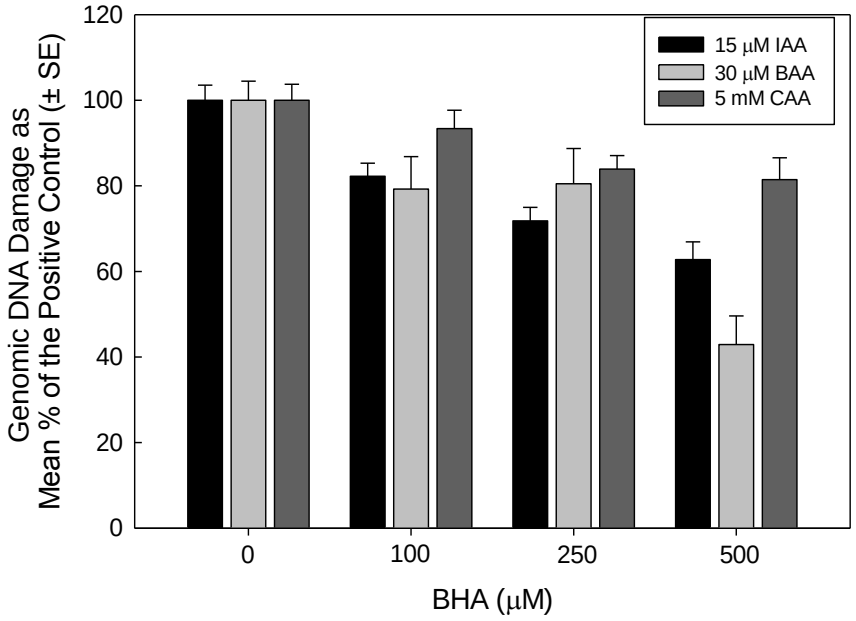

Figure 4.1. CHO cell genomic DNA damage after 4 h treatment with monoHAA ± BHA. Data for each monoHAA are presented as percentages of their respective positive control values.

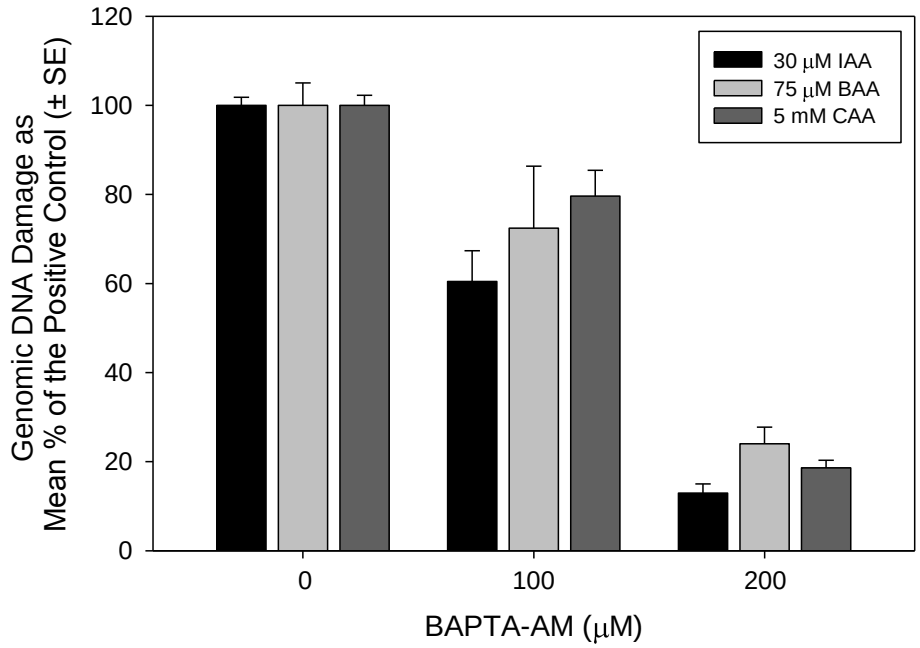

Figure 4.2. CHO cell genomic DNA damage after 4 h treatment with monoHAA ± BAPTA-AM. Data for each monoHAA are presented as percentages of their respective positive control values.

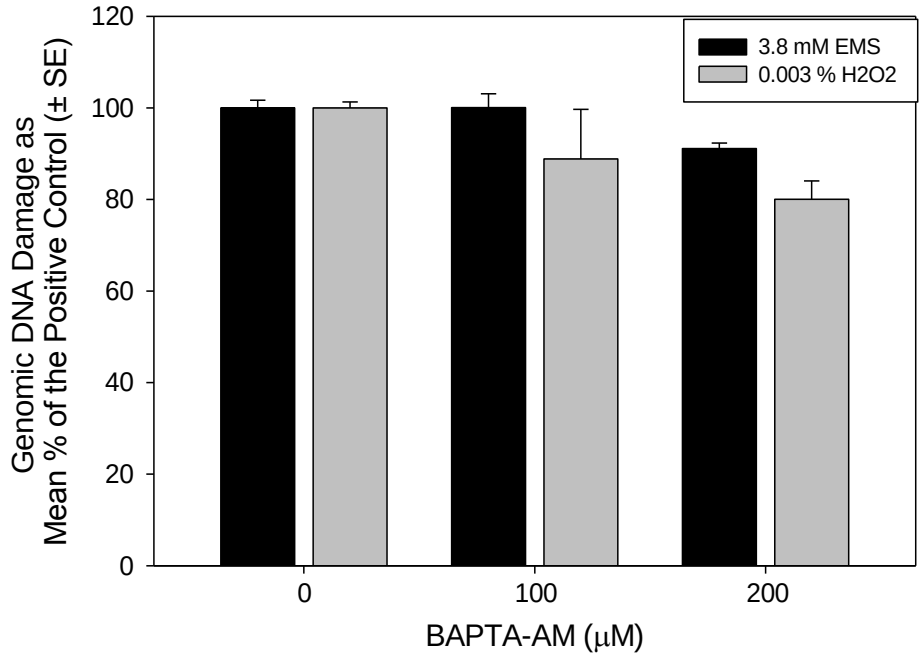

Figure 4.3. CHO cell genomic DNA damage after 4 h treatment with EMS ± BAPTA-AM or H_2O_2 ± BAPTA-AM. Data for EMS and H_2O_2 are presented as percentages of their respective positive control values.

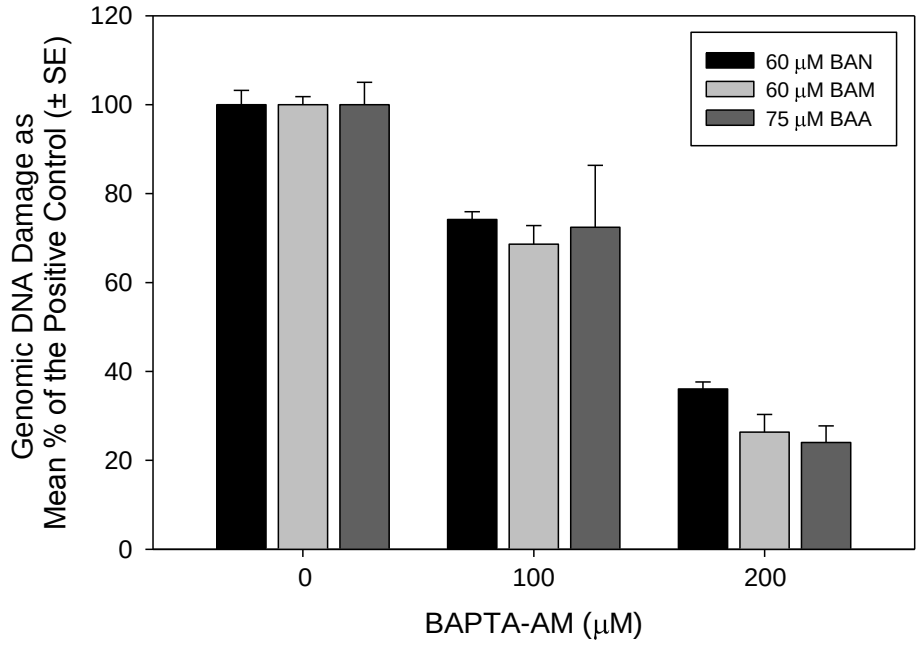

Figure 4.4. CHO cell genomic DNA damage after 4 h treatment with DBP ± BAPTA-AM. Data for each DBP are presented as percentages of their respective positive control values.

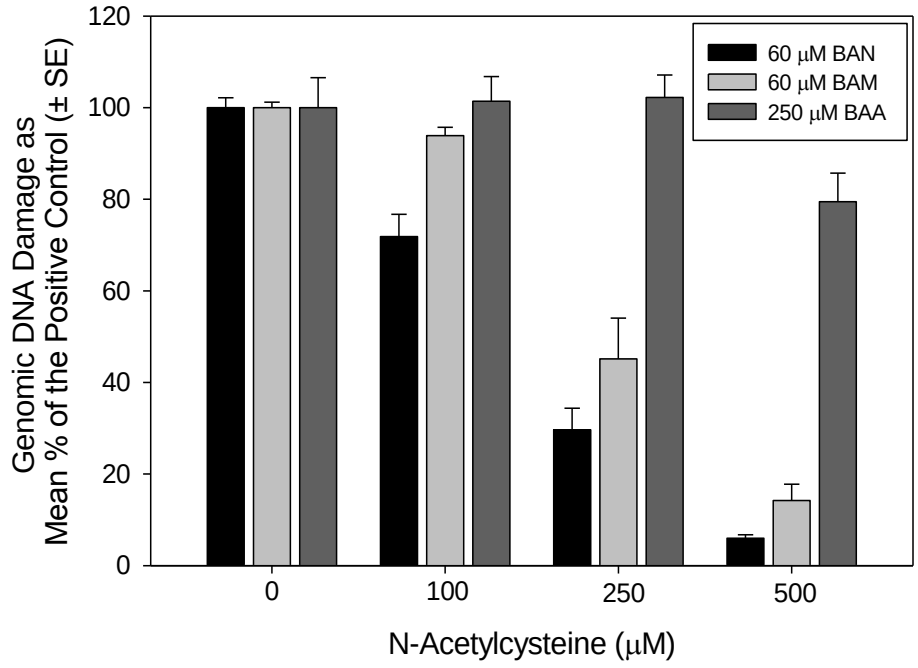

Figure 4.5. CHO cell genomic DNA damage after 4 h treatment with DBP ± NAC. Data for each DBP are presented as percentages of their respective positive control values.

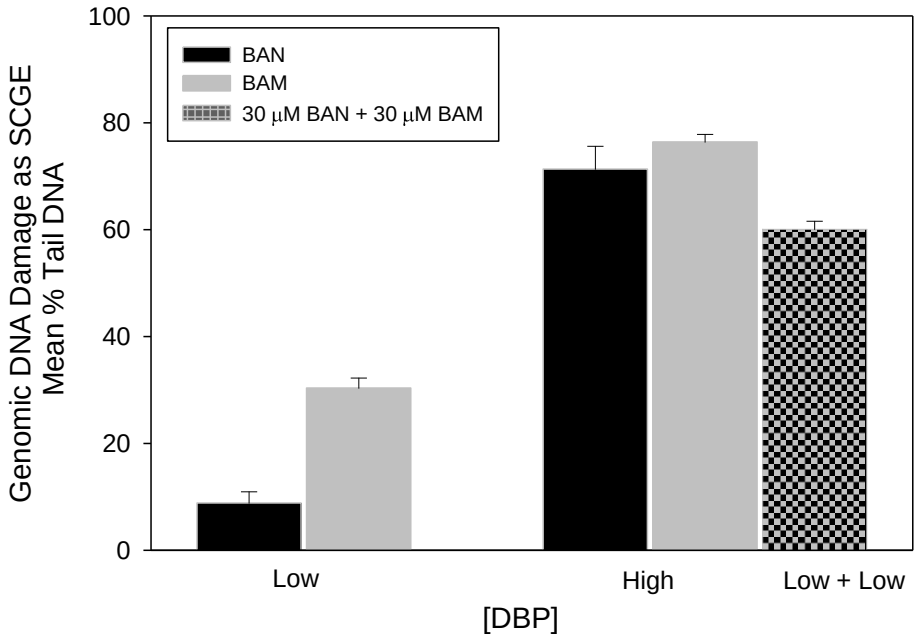

Figure 4.6. CHO cell genomic DNA damage after 4 h treatment with BAN, BAM or BAN + BAM. For BAN and BAM Low concentration = 30 μM, High concentration = 60 μM, and the Low + Low concentration is a mixture of 30 μM BAN and 30 μM BAM.

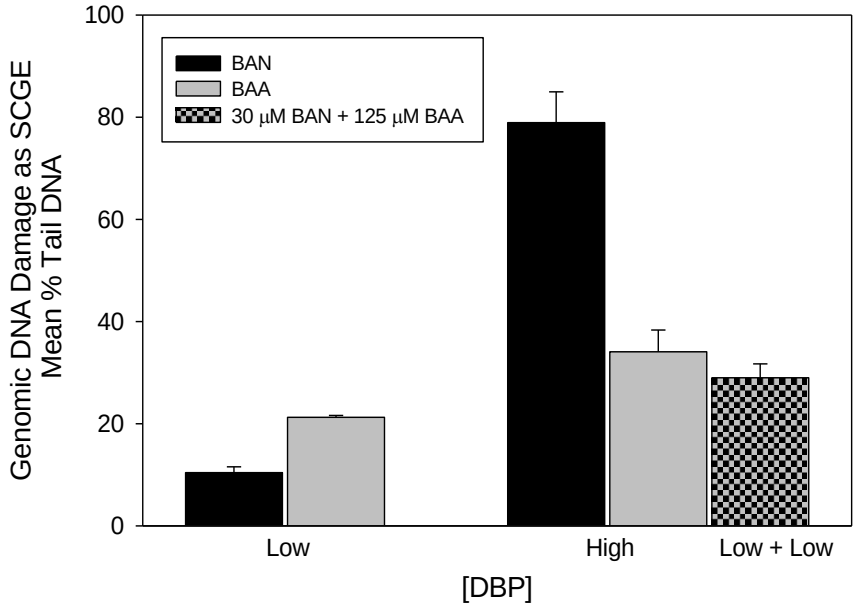

Figure 4.7. CHO cell genomic DNA damage after 4 h treatment with BAN, BAA or BAN + BAA. For BAN and BAA Low concentration = 30 µM and 125 µM respectively, High concentration = 60 µM and 250 µM respectively, and the Low + Low concentration is a mixture of 30 µM BAN and 125 µM BAA.

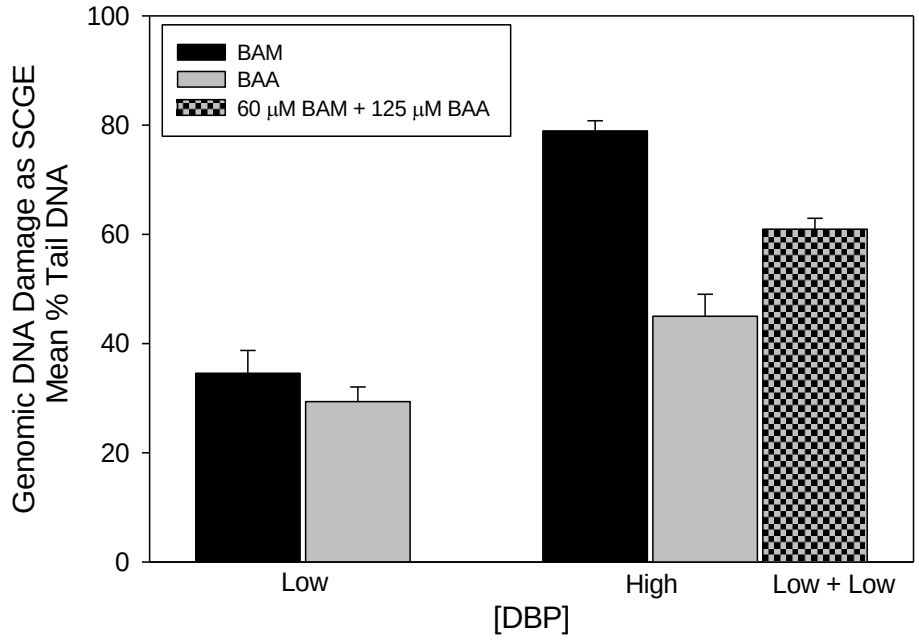

Figure 4.8. CHO cell genomic DNA damage after 4 h treatment with BAM, BAA or BAM + BAA. For BAM and BAA Low concentration = 30 μM and 125 μM respectively, High concentration = 60 μM and 250 μM respectively, and the Low + Low concentration is a mixture of 30 μM BAM and 125 μM BAA.

CHAPTER 5: CONCLUSIONS

Genotoxicity induced by the monoHAAs, followed the pattern of descending toxicity IAA > BAA > CAA. This pattern was shared by the alkylation potential of these compounds. Based on these observations a working hypothesis was formed, in which the monoHAAs damaged DNA by direct alkylation. However, experiments in which antioxidants reduced the genotoxicity and mutagenicity of IAA and toxicogenomic analyses that showed cells treated with the monoHAAs were responding to double strand breaks suggested that ROS, rather than direct alkylation was responsible for their genotoxicity. Based on a review of the neurotoxicology literature involving IAA, disruption of glycolysis via the inhibition of GAPDH emerged as a possible pathway through which DNA could be damaged in a manner that fit well with the emerging data. The research described in this dissertation was conducted with the aim of systematically testing a new working hypothesis in which monoHAAs inhibit glycolysis, increase intracellular Ca^{2+}, and generate ROS which are ultimately responsible for genotoxicity.

The ability of the monoHAAs to interact directly with DNA isolated from the cellular environment was investigated. While each of the monoHAAs generated DNA damage in treated cells, acellular DNA was not vulnerable to alkylation by these compounds. This finding solidified growing doubts that direct alkylation was the mode of action by which the monoHAAs were damaging DNA, and suggested some cellular target(s) remained to be discovered. A comparative study evaluating the effect of each monoHAA on GAPDH activity showed that the ability to inhibit this enzyme closely followed the pattern of toxicity, where IAA > BAA > CAA. A correlation analysis revealed that GAPDH inhibition potential was strongly associated with toxicological endpoints including cytotoxicity, genotoxicity, mutagenicity, and teratogenicity.

Inhibition of glycolysis disrupts Ca^{2+} homeostasis which can generate ROS. Thus the working hypothesis grew to include a link between inhibition of GAPDH and formation of ROS involving disruption of Ca^{2+}. A series of studies were conducted to investigate if each of the monoHAAs could generate ROS in non-neuronal cell lines, and if they were involved in damaging DNA. Each of the monoHAAs activated ARE driven transcription of a reporter gene and modulated transcription levels of oxidative stress responsive genes, two sensitive indicators of oxidative stress. BHA mitigated the genotoxicity of each of the monoHAAs and provided evidence that DNA damage was resulting from ROS. Buffering the cytosolic Ca^{2+} levels with BAPTA-AM also reduced DNA damage induced by the monoHAAs.

These data helped to solidify the working hypothesis (Figure 5.1), and strongly suggested that a cascade of events leading to genomic DNA damage could be initiated by the inhibition of GAPDH. However, the goal of identifying the toxic mechanisms for individual DBPs should ultimately be to understand how multiple chemicals can interact to generate toxicity. DBP exposure was linked to adverse health and pregnancy outcomes, but because many individual DBPs are generated in small, likely non-toxic amounts, understanding possible interactions among individual chemicals might provide a better understanding of the cumulative effect of exposure. With this goal in mind, another set of experiments was designed to investigate the genotoxicity of defined component DBP mixtures. BAN and BAM were chosen because oxidative stress and disruption of Ca^{2+} homeostasis were associated with their toxicities. Buffering cytosolic Ca^{2+} levels with BAPTA-AM or supplementing the GSH precursor NAC significantly attenuated genotoxicity induced by BAN and BAM; these data showed that Ca^{2+} and ROS were involved in DNA damage generated by these compounds. Combining BAA with either BAN or BAM produced an additive genotoxic effect. This finding was interesting in that

while each of BAA, BAN, and BAM share disruption of Ca^{2+} homeostasis and oxidative stress in their toxic cascades, the initiating events are different (GSH depletion for BAN and BAM; inhibition of GAPDH for BAA). The additive genotoxic effect of these compounds suggested that multiple compounds could converge through different pathways to generate toxicity. The monoHAAs, BAN, and BAM are soft electrophiles that react preferentially with soft nucleophiles, which, in biological systems, include cysteine thiol moieties. Cysteine thiols play an important role in redox regulation; an assault of soft electrophiles could overwhelm the cellular redox potential and generate oxidative stress through multiple pathways leading to cancer, neurodegenerative disease, or any of the many associated pathologies.

Further investigation of the individual mechanisms of toxicity and interactions among DBPs will provide the necessary information to mitigate the health and reproductive risks associated with disinfected water. By identifying the mechanisms leading to adverse health and reproductive outcomes water treatment processes can be engineered to either eliminate the DBP precursors in the source water or target specific DBPs or DBP classes for removal from the distribution systems. Additionally, biomarkers of oxidative stress could be used to mark DBP exposures and assays could be developed to measure the overall effect of disinfected water so that Federal regulations and water treatment processes can be focused on reducing oxidative stress as a way to prevent adverse health and reproductive outcomes associated with DBPs.

The major findings presented within this dissertation include:

- MonoHAAs initiate a genotoxic cascade by inhibiting glycolysis, which disrupts Ca^{2+} homeostasis and generates oxidative stress.

- BAN and BAM deplete cellular GSH disrupt Ca^{2+} homeostasis and generates oxidative stress.
- DBPs that generate oxidative stress have a cumulative genotoxic effect.
- Transcription of COX-2 was upregulated by 4 h monoHAA exposures; this uncovered an important link between cancer and DBP exposure.
- Two possible Ca^{2+} dependent sources of ROS were identified in NOX5 and COX-2.

Figure

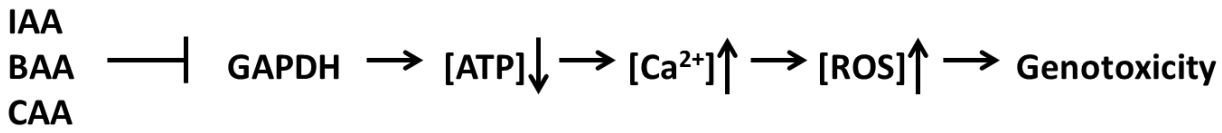

Figure 5.1. Genotoxic cascade initiated by monoHAA induced inhibition of GAPDH.