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Monohalogenated acetamide-induced cellular stress and genotoxicity are related to electrophilic softness and thiol/thiolate reactivity

Justin A. Pals[1,2], *Elizabeth D. Wagner*[1,3], *Michael J. Plewa*[1,3], *Menghang Xia*[4], *Matias S. Attene-Ramos*[2,*]

1. *Department of Crop Sciences, University of Illinois at Urbana-Champaign, Urbana, IL, United States*
2. *Department of Environmental and Occupational Health, Milken Institute School of Public Health, George Washington University, Washington, DC, United States*
3. *Safe Global Water Institute, University of Illinois at Urbana-Champaign, Urbana, IL, United States*
4. *National Center for Advancing Translational Sciences, National Institutes of Health (NIH), Department of Health and Human Services (DHHS), Bethesda, MD, United States*

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ABSTRACT

Haloacetamides (HAMs) are cytotoxic, genotoxic, and mutagenic byproducts of drinking water disinfection. They are soft electrophilic compounds that form covalent bonds with the free thiol/thiolate in cysteine residues through an S_N2 reaction mechanism. Toxicity of the monohalogenated HAMs (iodoacetamide, IAM; bromoacetamide, BAM; or chloroacetamide, CAM) varied depending on the halogen substituent. The aim of this research was to investigate how the halogen atom affects the reactivity and toxicological properties of HAMs, measured as induction of oxidative/electrophilic stress response and genotoxicity. Additionally, we wanted to determine how well *in silico* estimates of electrophilic softness matched thiol/thiolate reactivity and *in vitro* toxicological endpoints. Each of the HAMs significantly induced nuclear Rad51 accumulation and ARE signaling activity compared to a negative control. The rank order of effect was IAM > BAM > CAM for Rad51, and BAM ≈ IAM > CAM for ARE. In general, electrophilic softness and *in chemico* thiol/thiolate reactivity provided a qualitative indicator of toxicity, as the softer electrophiles IAM and BAM were more thiol/thiolate reactive and were more toxic than CAM.

Introduction

Disinfection of municipal drinking water is an essential safeguard of public health (Calderon, 2000). However, the addition of disinfectants to source waters containing organic and inorganic precursor molecules generates a mixture of toxic halogenated byproducts (Krasner et al., 1989). Many of these disinfection byproducts (DBPs) are cytotoxic, genotoxic, and mutagenic *in vitro*, and some are carcinogenic in rodents (Richardson et al., 2007). Moreover, epidemiologic studies suggested long-term exposures to DBPs increased risk of bladder cancer (Michaud et al., 2007; Villanueva et al., 2007). Still, the mechanisms of toxicity leading to the observed adverse effects are not fully understood, making it difficult to establish effective interventions. Additionally, since DBPs are formed at concentrations below their individual adverse effect levels, it is unlikely that any

* *Corresponding author*. E-mail: attene@gwu.edu (Matias S. Attene-Ramos).

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individual DBP can account for the aforementioned observed adverse effects highlighting the need for understanding the toxic effect of the whole mixture (Bull, 2006; Simmons et al., 2002). Identification of chemical groups that share a common mechanism within the DBP mixture would provide a better understanding of the mixture toxicity (U.S. EPA, 2002) and could provide valuable insight on how to improve chemical regulation.

Several DBPs can be classified as soft electrophilic compounds (highly polarizable chemicals deficient in electrons) that preferentially react with biological soft nucleophiles (*e.g.*, thiol/thiolate groups) forming covalent bonds (Pearson, 1963). For example, many DBPs share an α-brominated carbonyl motif identified as a structural predictor of thiol (glutathione (GSH)) reactivity (Hughes et al., 2015). Additionally, cytotoxicity and genotoxicity correlated with predicted S_N2 reactivity for several α-halogenated carbonyl (or nitrile) containing DBP chemical classes including haloacetonitriles (HANs), haloacetamides (HAMs), and haloacetic acids (HAAs) (Muellner et al., 2007; Plewa et al., 2008, 2010). Genotoxicity induced by the α-halogenated carbonyl (or nitrile) containing DBPs, bromoacetamide (BAM), and bromoacetonitrile (BAN) were significantly reduced by co-treating with N-acetylcysteine (NAC) (Pals et al., 2016). Therefore, reactivity seems to play a significant role in the toxicity of DBPs, where directly perturbing the intracellular thiol pool through alkylation of cellular thiol/thiolates leads to a toxic response by disrupting various cellular processes (Schultz et al., 2006). Moreover, GSH depletion is observed in the progression of several diseases including cancer, cardiovascular, and neurodegenerative diseases (Ballatori et al., 2009).

Furthermore, reactive electrophiles with common cellular targets present an ideal opportunity for additive or synergistic toxicity (U.S. EPA, 2002). In fact, experiments with α,β-unsaturated carbonyl derivatives, showed additive toxicity in binary and ternary mixtures *in vitro* and in an *in vivo* model (Zhang et al., 2016). Work by Dawson and colleagues showed that HANs and halogenated ethyl acetates (α-halogenated carbonyl containing compounds) generated, in some cases, dose additive toxicity within and among these chemical classes (Dawson et al., 2010, 2011, 2014). Additive genotoxicity was observed in binary mixtures of BAM and BAN (Pals et al., 2016).

Soft electrophile reactivity can be predicted using *in silico* methods based on Hard Soft Acid Base (HSAB) theory (Karelson et al., 1996). HSAB theory makes use of frontier molecular orbital (FMO) energies to predict reactivity between electrophile–nucleophile pairs (Karelson et al., 1996). Several parameters for reactivity based on FMO energies were developed and utilized in Quantitative Structure Activity Relationship (QSAR) applications (Schwöbel et al., 2011); however, in its basic form, HSAB theory suggests that an electrophile is soft if it has a low energy lowest unoccupied molecular orbital (E_{LUMO}) (Karelson et al., 1996). Estimations of E_{LUMO}, therefore, provide a theoretical method for identifying soft electrophiles among identified DBPs. Our previous work showed that E_{LUMO} was a useful predictor of thiol reactivity and genotoxicity in a set of mono-brominated DBPs (bromoacetic acid, BAM, and BAN) (Pals et al., 2016). However, because reactivity of alkyl halides is dependent on halogen leaving efficiency, the effect of variable halogen substitution requires additional investigation. The set of monoHAMs, iodoacetamide (IAM), BAM, and chloroacetamide (CAM), isolates the effect the halogen substituent asserts on thiol/thiolate reactivity and toxicity, and allows us to evaluate *in silico* softness parameters as predictors of these effects. In this study we measured the ability of each monoHAM to generate an Antioxidant Response Element (ARE) driven stress response, and to generate toxicity as DNA double strand breaks. We then compared toxic potencies with *in chemico* reactivity and *in silico* parameters derived from FMO energies to determine their ability to predict *in vitro* effects.

1. Experimental

1.1. General reagents

General laboratory reagents were purchased from Fisher Scientific Co. (Itasca, IL) or Sigma Aldrich Co. (St. Louis, MO). IAM, BAM, CAM, DTNB (5,5′-dithiobis (2-nitrobenzoic acid)) and NAC were purchased from Sigma-Aldrich (St. Louis, MO).

1.2. In silico estimates of FMO energies

FMO energies, including energy of the highest occupied molecular orbital (E_{HOMO}) and E_{LUMO} were estimated using density function B3LYP 6-311+G**, from Hartree Fock 6-311+G** equilibrium geometries with Spartan 10 software (Wavefunction Inc., Irvine, CA). Electrophilic hardness (η), softness (σ), chemical potential (μ), and electrophilic index (ω) were calculated using Eqs. (1)–(4) respectively.

$$\text{Hardness } (\eta) = [E_{LUMO} - E_{HOMO}]/2 \quad (1)$$

$$\text{Softness } (\sigma) = 1/\eta \quad (2)$$

$$\text{Chemical Potential } (\mu) = [E_{LUMO} + E_{HOMO}]/2 \quad (3)$$

$$\text{Electrophilic Index } (\omega) = \mu^2/2\eta \quad (4)$$

1.3. NAC reactivity

NAC served as a model soft nucleophile to determine HAM electrophilic reactivity. After exposure to the HAMs (30 min), the remaining free thiol was quantified with Ellman's reagent (Ellman, 1959) with minor modifications of the previously published protocol (Pals et al., 2016). For these experiments, Ellman's reagent was prepared from stock solutions of 100 mM DTNB in dimethyl sulfoxide (DMSO), and 200 mM EDTA (ethylenediaminetetraacetic acid) in deionized water diluted to 1 and 0.1 mM, respectively, in 200 mM Tris buffered deionized water (pH 8.0). Fresh 1 M stock solutions of NAC or HAM were prepared in DMSO for each experiment. HAMs and NAC were further diluted into 200 mM Tris pH 8.0. Each HAM, in a range of concentrations from 0 to 2000 μM, was mixed with 400 μM NAC in a total volume of 50 μL 200 mM Tris pH 8.0 in a 96 well microplate. Reactions occurred at room temperature with orbital shaking at 250 r/min. After the reaction time expired, 50 μL of Ellman's reagent was added to the wells. After 3 min at 250 r/min the absorbance at 412 nm (A_{412}) was measured for each well with a Spectramax Paradigm plate reader (Molecular Devices, Sunnyvale, CA).

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