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Energy of the Lowest Unoccupied Molecular Orbital, Thiol Reactivity, and Toxicity of Three Monobrominated Water Disinfection Byproducts

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Abstract

Disinfection of drinking water protects public health against waterborne pathogens. However, during disinfection, toxic disinfection byproducts (DBPs) are formed. Exposure to DBPs was associated with increased risk of bladder cancer in humans. DBPs are generated at concentrations below their carcinogenic potencies; it is unclear how exposure leads to adverse health outcomes. We used computational estimates of the energy of the lowest unoccupied molecular orbital (E_{LUMO}) to predict thiol reactivity and additive toxicity among soft electrophile DBPs. Bromoacetic acid (BAA) was identified as non-thiol-reactive, which was supported by *in chemico* and *in vitro* data. Bromoacetonitrile (BAN) and bromoacetamide (BAM) were thiol-reactive. Genotoxicity induced by these compounds was reduced by increasing the thiol pool with *N*-acetyl L-cysteine (NAC), while NAC had little effect on BAA. BAN and BAM shared depletion of glutathione (GSH) or cellular thiols as a molecular initiating event (MIE), whereas BAA induces toxicity through another pathway. Binary mixtures of BAM and BAN expressed a potentiating effect in genotoxicity. We found that soft electrophile DBPs could be an important predictor of common mechanism groups that demonstrated additive toxicity. *In silico* estimates of E_{LUMO} could be used to identify the most relevant DBPs that are the forcing factors of the toxicity of finished drinking waters.

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SCGE, statistical analyses, and references (PDF)

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Introduction

Disinfection of municipal water greatly improved public health by reducing diseases transmitted through drinking water.¹ Disinfectants, such as chlorine, chloramines, chlorine dioxide, or ozone, kill pathogenic microorganisms but also react with organic and inorganic materials, forming toxic disinfection byproducts (DBPs) in drinking water as an unintended consequence. Since they were first discovered in 1974,^{2,3} over 600 individual DBPs have been identified in disinfected water at relatively low concentrations (from low micrograms per liter to nanograms per liter).⁴

Long-term exposure to DBPs was associated with a significant increased risk for bladder cancers.⁵ Although individual DBPs were genotoxic and mutagenic *in vitro* and some were carcinogenic in rodent assays,⁶ the toxicity of these individual DBPs does not account for the increased human risk of cancer; the concentrations required in laboratory animals would not be achieved by drinking, bathing, and/or swimming in disinfected water.⁷ This disparity between toxicology and human risk suggests that multiple DBPs contribute to the overall toxicity. The United States Environmental Protection Agency (U.S. EPA) guidance for mixture toxicity suggests that chemicals that act through the same mechanism generate dose additive toxicity.⁸ Identifying mechanisms of toxicity for DBPs and sorting them into common mechanism groups (CMGs) would provide a better understanding of the toxicity of the mixture of DBPs in drinking water.

In a systematic quantitative evaluation of DBP toxicity using a Chinese hamster ovary (CHO) model cell line, monohalogenated haloacetic acids (monoHAAs),⁹ haloacetonitriles (monoHANs),¹⁰ and haloacetamides (monoHAMs)¹¹ were among the most genotoxic. Within each of these chemical classes, S_N2 reactivity, driven largely by the leaving efficiency of the halogen substituent, correlated with toxicity, suggesting a reactive mechanism of toxicity.^{9–11} Dawson et al. investigated cumulative toxicity of S_N2 -reactive haloacetonitriles (HANs) and ethyl- α -halogenated acetates and reported some instances of dose additive toxicity within and among these chemical classes.^{12–14} The electrophiles within these classes reacted with glutathione (GSH) *in chemico*; the reported dose additive toxicity may be attributed to collective GSH depletion as a shared mechanism or molecular initiating event (MIE).^{12–14} Investigating S_N2 -reactive compounds or GSH-depleting agents as a CMG may aid in evaluating additive toxicity within complex DBP mixtures. Many of

the genotoxic DBP chemical classes, including the HANs,¹⁰ haloacetamides (HAMs),¹¹ and haloacetic acids (HAAs),¹⁵ expressed correlations between genotoxicity and S_N2 reactivity; furthermore, many DBPs, including these chemical classes, are known to react with or deplete GSH and generate oxidative stress.^{16–20}

The activated primary alkyl halide functional group, predicted as GSH-reactive, is present in many DBPs and generates a relatively soft electrophilic center.²¹ In biological systems, the predominant soft nucleophile is the thiol moiety found in cysteine. GSH, which is often present in cells at millimolar concentrations, provides a thiol buffer or threshold for soft electrophile toxicity in addition to its role as an antioxidant.²² Toxic responses to DBP mixtures may arise from collective soft electrophile depletion of cellular GSH or thiol pools, leading to electrophilic and oxidative stress. Thus, thiol reactivity may be a useful tool in investigating the cumulative toxicity of the total DBP exposure. GSH reactivity *in chemico* served as a suitable predictor of thiol reactivity;^{12,13} however, with over 600 individual DBPs identified, a more efficient method for predicting thiol reactivity is needed. Hughes et al. demonstrated that the energy of the lowest unoccupied molecular orbital (E_{LUMO}) was a major predictor of reactive site(s) within a molecule and also GSH reactivity.²¹ They identified the α -halogenated carbonyl as a structural motif predictive of GSH reactivity.²¹ Furthermore, the hard–soft acid base (HSAB) theory uses frontier molecular orbital (FMO) energies, including E_{LUMO} , to predict reactivity among electrophile and nucleophile pairs.²³ In general, electrophiles with low E_{LUMO} values are soft electrophiles that react preferentially with soft nucleophiles, of which the thiol moiety present in cysteine is the softest in biological systems.²³ Thus, E_{LUMO} may serve as a parameter for identifying soft electrophiles and predicting toxicity derived from thiol depletion.

Toxicants that depleted GSH caused protein misfolding, disrupted calcium (Ca^{2+}) homeostasis, and oxidative stress.²⁴ Although the direct link between DBP exposure and adverse health outcomes is not well understood, oxidative stress and genotoxicity were suggested as possible mechanisms.²⁵ The monoHAAs and iodoacetamide (IAM) generated Ca^{2+} -dependent oxidative stress.^{17,26,27} Buffering intracellular Ca^{2+} with ethylene glycol-bis(β -aminoethyl)- N,N,N',N' -tetraacetoxymethyl ester (EGTA-AM)¹⁷ or 1,2-bis(2-aminophenoxy)-ethane- N,N,N',N' -tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM)²⁸ reduced toxicity generated by IAM and iodoacetate (IOA), respectively. The intracellular calcium chelator BAPTA-AM also reduced the generation of reactive oxygen species (ROS) in iodoacetate-treated neurons.²⁸

While IAA- and IAM-treated cells expressed increased intracellular Ca^{2+} and the generation of ROS, different MIEs were proposed for their toxic effects. IAA inhibited the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH).¹⁸ GAPDH inhibition strongly correlated with multiple toxicological end points for each monoHAA and was identified as the MIE for these DBPs.²⁹ IAM weakly inhibited GAPDH but reacted more readily with GSH,¹⁸ and depletion of this intracellular antioxidant and electrophile-scavenging tripeptide is its likely MIE.²⁴ The monoHANs are structurally similar to the monoHAAs and monoHAMs, and chloroacetonitrile (CAN) reacted directly with GSH.¹² ^{14}C -labeled CAN was detected in DNA; however, this reactivity with DNA was dependent upon GSH depletion.³⁰ GSH depletion and induction of oxidative stress were

important initiators of toxicity for CAN under physiological GSH concentrations.¹⁶ These studies demonstrate that the resulting ROS-induced toxicity from these DBP classes are based on different MIEs.

The purpose of this research was to determine if *in silico* E_{LUMO} estimation could predict thiol reactivity *in chemico* and *in vitro* and if E_{LUMO} could predict mechanism(s) of toxicity. We selected model brominated DBPs [bromoacetic acid (BAA), bromoacetamide (BAM), and bromoacetonitrile (BAN)] that share a common reactive site with similar geometry. We investigated the roles that intracellular GSH and Ca^{2+} levels play in the MIEs for toxicity of these model DBPs.

Materials and Methods

General Reagents

General laboratory reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) or Fisher Scientific Co. (Itasca, IL). BAA was purchased from Fluka Chemical Co. (Buchs, Switzerland). BAM (98%), BAN (97%), *N*-acetyl L-cysteine (NAC), BAPTA-AM, and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture medium (Ham's F12) and fetal bovine serum (FBS) were purchased from Fisher Scientific Co. Stock solutions of 1 M (BAA, BAM, BAN, and NAC) or 50 mM (BAPTA-AM) were prepared in dimethyl sulfoxide (DMSO) and stored in sterile glass vials at -22°C . Stock solutions were diluted in F12 immediately prior to use.

Chinese Hamster Ovary (CHO) Cells

CHO cell line AS52, clone 11-4-8, served as a mammalian cell model for *in vitro* experiments.³¹ CHO cells were maintained in Ham's F12 culture medium supplemented with 5% FBS, 1% antibiotic (100 units/mL sodium penicillin G, 100 $\mu\text{g/mL}$ streptomycin sulfate, and 0.25 $\mu\text{g/mL}$ amphotericin B in 0.85% saline), and 1% glutamine at 37°C in a humidified atmosphere of 5% CO_2 .

Computational Estimations of E_{LUMO} and LUMO Mapping

Computations were performed using Spartan (version 5.0) software. Equilibrium geometries for each test agent were calculated using the Hartree–Fock 3-21G basis set. From the equilibrium geometry, energy including E_{LUMO} and energy of the highest occupied molecular orbital (E_{HOMO}) were estimated using the density function B3LYP 6-31G* basis set. LUMO maps were generated for each compound to determine the site of reactivity, and E_{LUMO} was used as a parameter to evaluate thiol reactivity among the agents.

In Chemico Free Thiol Reactivity

To quantify free thiol reactivity for BAA, BAM, and BAN, each DBP (0–2000 μM) was reacted with NAC (400 μM) in a total volume of 50 μL of 200 mM Tris buffer at pH 8.0 in a 96-well microplate. The reaction occurred at room temperature with shaking (200 rpm) for 20 min. After the reaction time, 50 μL of Ellman's reagent [1 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 100 mM KPO_3 buffer + 0.1 mM EDTA] was added, for a total volume of 100 μL and a final NAC concentration of 200 μM . An absorbance measurement at 412

nm was recorded for each well using a Spectramax Paradigm plate reader (Molecular Devices). The data were blank-corrected using A_{412} measurements from a blank reaction well (Tris, DBP, and Ellman's reagent). The reaction wells with 200 μ M NAC + 0 μ M DBP served as the maximum (100%) free thiol A_{412} value. The remaining data were normalized to and reported as a percentage of the 100% A_{412} value.

GSH Reactivity *In Vitro*

As an *in vitro* measure of thiol alkylation by each model DBP, total GSH (GSH + GSSG = GSx) was measured using the GSH-Glo kit (Promega) according to the protocol of the manufacturer, with a minor modification. Prior to treatment, 5×10^3 CHO cells per well were seeded onto opaque white 96-well microplates (Costar). To evenly distribute cells in the wells, the plates were rocked 10 min, with a 90° rotation after the initial 5 min, and then incubated overnight at 37 °C in a 5% CO₂ humidified atmosphere. The next day, the medium was removed by aspiration and the cells were washed once with 100 μ L of Hank's balanced salt solution (HBSS). DBP concentrations were chosen that induced approximately equivalent genotoxic responses. Cells were treated for 2 h in 25 μ L of F12 medium. After treatment, the medium was aspirated and 100 μ L of room-temperature GSH Glow reagent, prepared according to the protocol of the manufacturer, was added to each well. The microplate was put in an orbital shaker for 5 min at 200 rpm. The microplate was allowed to equilibrate at room temperature for 25 min. To reduce oxidized GSH (GSSG), 10.1 μ L of 1 mM dithiothreitol (DTT) was added to each well (final concentration of 100 μ M DTT) and the microplate was shaken at 200 rpm for 5 min. To each well, 100 μ L of Luciferin Detection Reagent was added. The microplate was shaken for 5 min at 200 rpm and equilibrated at room temperature for 10 min. Luminescence, with a 250 ms integration time, was measured using a Spectramax Paradigm plate reader.

Single-Cell Gel Electrophoresis (SCGE)

SCGE was used to measure the genotoxicity of the DBPs and the effect of NAC or BAPTA-AM on the genotoxicity induced by these DBPs. Additionally, we used SCGE to measure the genotoxicity of DBP binary mixtures. The SCGE (Comet) assay quantitatively measures genomic DNA damage in individual nuclei induced by a test agent.³² We employed a microplate methodology.³³ The SCGE metric for induced genomic DNA damage was the percent tail DNA value, which is the amount of DNA that migrated from the nucleus into the microgel.³⁴ Within each concentration range with >70% cell viability, a concentration–response curve was generated.

Statistical Analyses

To compare the data for significant differences against their controls, a one-way analysis of variance (ANOVA) test was employed using SigmaPlot, version 13 (Systat Software, Inc.). If a significant *F* value ($p < 0.05$) was obtained, a Holm–Sidak multiple comparison test was performed. For the SCGE assay, the percent tail DNA values are not normally distributed. The mean percent tail DNA value for each microgel was calculated, and these values were averaged among all of the microgels within each treatment group. An ANOVA test was conducted on these averaged mean percent tail DNA values. If a significant *F* value of p

0.05 was obtained, a Holm–Sidak multiple comparison versus the control group analysis was conducted with the power of 0.8 at $\alpha = 0.05$.

Results and Discussion

Reactivity of electrophile/nucleophile pairs can be predicted by the HSAB theory. The activated primary alkyl halide site of reactivity common among the HAAs, HAMs, and HANs makes these compounds relatively soft electrophiles; thus, their toxicity could be derived from reacting with cellular thiols. Because the predictive ability of the HSAB theory is largely driven by FMO energies, we used computational estimates of E_{LUMO} and LUMO mapping of the reactive site(s) to investigate the MIEs of the monobrominated species of three genotoxic DBP chemical classes.

On the basis of previous studies,^{12–14} we hypothesized that chemicals with similar MIEs will produce a cumulative effect in mixtures. If soft electrophiles generate toxicity by depleting GSH or the cellular thiol pool, then the thiol pool acts as a threshold for toxicity. Any single DBP that alkylates and depletes GSH or the cellular thiol pool should potentiate the toxicity of any other DBP that acts through the same mechanism. Because a fraction of the cellular threshold is depleted by each soft electrophile in a mixture of DBPs, the toxicity of any GSH reactive compound is potentiated. We tested this hypothesis by overlapping computational chemistry and quantitative toxicological approaches.

Site of Reactivity and E_{LUMO} : *In Silico* Predictors of Thiol Reactivity

$\text{S}_{\text{N}}2$ reactions involve electron transfer from an electron-rich nucleophile to an electron-deficient electrophile. The reactivity of electrophile/nucleophile pairs can be estimated using estimations of the energy of the FMOs, because HOMO and LUMO are the orbitals that participate in electron transfer in the bimolecular reaction.³⁵ E_{LUMO} can also be used to define the site of reactivity within an electrophilic molecule.²¹ We defined these parameters and used them to investigate MIEs for three brominated DBPs (Br-DBPs).

FMO parameters and site of reactivity have been explored as predictors of thiol or GSH reactivity.²¹ E_{LUMO} was a major contributor to predicting GSH reactivity among pharmacological compounds.²¹ The predictive nature of E_{LUMO} can be explained in part by the HSAB theory, which suggests that soft electrophiles preferentially react with soft nucleophiles and hard electrophiles react primarily with hard nucleophiles. Spartan software was employed to calculate E_{LUMO} and also map the LUMO onto the selected Br-DBPs. The LUMO maps for BAA, BAN, and BAM are presented in Figure 1. For comparison, the LUMO map for bromoacetaldehyde is provided (Figure 1). For BAA, BAM, and BAN, the LUMO density was distributed to varying degrees between the activated primary alkyl halide and the α carbon. BAA and BAN clearly showed the LUMO density centered on the α carbon. The LUMO density of BAM was distributed across its two carbons, but the LUMO map indicated that the α carbon is the site of reactivity. The site of reactivity explains the $\text{I} > \text{Br} \gg \text{Cl}$ pattern of cytotoxicity and genotoxicity for the monohalogenated acetic acids,³⁶ acetamides,¹¹ and acetonitriles,¹⁰ because the $\text{S}_{\text{N}}2$ reactivity of the compound is dependent upon the leaving efficiency of the halogen substituent. In contrast, bromoacetaldehyde has the LUMO density centered on the carbonyl carbon, suggesting that

the aldehyde functional group is the most reactive site. Interestingly, the monohalogenated aldehydes do not exhibit the same $I > Br \gg Cl$ pattern of cytotoxicity and genotoxicity;³⁷ this was consistent with the reactive site of the molecule being independent of the halogen.

While the site of reactivity for BAA, BAM, and BAN was predicted to be the same primary alkyl halide functional group, the predicted E_{LUMO} value for BAA [specifically the bromoacetate anion species ($pK_a \sim 2.9$) that is dominant at physiological pH] is much higher than that in BAM or BAN (Table 1).

***In Chemico* Thiol Reactivity**

Because S_N2 reactivity of an electrophile/nucleophile pair is dependent upon transfer of electrons from the nucleophile HOMO to electrophile LUMO, electrophile E_{LUMO} is a useful predictor of reactivity with a specific nucleophile. The alkyl halide functional group generally creates a soft electrophilic reactive center. Cysteine thiols are the predominant soft nucleophiles in biological systems; thus, we compared the E_{LUMO} values predicted *in silico* for BAA, BAM, and BAN to thiol reactivity measured *in chemico* to evaluate the utility of E_{LUMO} as a predictor of thiol reactivity.

Reactivity with the free thiol in NAC was measured for the Br-DBPs. Figure 2A illustrates the percent free thiol remaining after 20 min of reaction with BAA, BAM, or BAN. The lowest concentrations that significantly reduced total free thiol were 1500 μM BAA, 200 μM BAM, or 200 μM BAN.

The rank order of thiol reactivity based on the linear regression of the data illustrated in Figure 2A was $BAM > BAN \gg BAA$. The E_{LUMO} estimations did not match the rank order of thiol reactivity *in chemico*, with BAM having the strongest reactivity with NAC but BAN having the lowest estimated E_{LUMO} (Table 1). However, a strong but non-significant Pearson product moment correlation ($r = 0.89$) emerged. Additional data are needed to adequately investigate the relationship between E_{LUMO} and thiol reactivity; as the complexity of the molecule increases, additional factors, such as steric hindrance of the reactive site and leaving efficiency of the halogen substituent, will need to be addressed. However, our data were consistent with previous studies in finding *in silico* estimates of E_{LUMO} to be a predictor of thiol reactivity,²¹ and thus useful to predict or prioritize molecular nucleophile targets for electrophilic DBPs and electrophiles in general.

GSH Depletion: *In Vitro* Thiol Reactivity

A previous study showed differential GSH reactivity for IAA and IAM; IAM depleted GSx to a greater extent than IAA.¹⁸ While the LUMO map suggested a similar primary alkyl halide reactive moiety for BAA, BAM, and BAN, the E_{LUMO} values varied (Table 1). The *in chemico* thiol reactivity data presented in Figure 2A demonstrated that BAA was less reactive to the free thiol group in NAC.

Figure 2B presents GSx measured in CHO cells after 2 h of DBP exposure. The data were normalized to a concurrent negative control (cells without DBP exposure). All three Br-DBPs depleted GSx; however, BAN and BAM had a stronger effect. The E_{LUMO} value matched pairwise the GSx depletion *in vitro*. These data along with the *in chemico* data in

Figure 2A indicated that E_{LUMO} is a useful metric of thiol reactivity both *in chemico* and *in vitro* and can be used to predict the biological target of an electrophile.

Effect of NAC on Br-DBP-Induced Genotoxicity

The hypothesis that soft electrophile toxicity is derived from depletion of the cellular thiol pool was tested by exogenously increasing the available thiol pool with NAC. Approximately equivalent genotoxic concentrations (SCGE) of each Br-DBP (60 μM BAN, 60 μM BAM, or 250 μM BAA) served as positive controls; NAC was co-treated at 100, 250, and 500 μM . Percent tail DNA values for co-treated CHO cells are reported as a percentage of their respective positive controls (Figure 3). For BAN or BAM, a clear NAC concentration-dependent reduction of DNA damage was evident (Figure 3); significant ($p < 0.001$) reductions of DNA damage occurred with 100 and 250 μM NAC for BAN and BAM, respectively. The highest NAC concentration (500 μM) reduced DNA damage induced by BAA, BAM, or BAN by 20.6, 85.8, and 94.0%, respectively. With lower concentrations of NAC (100 and 250 μM), there was no significant protection against BAA-induced DNA damage, although there was a slight decrease with the highest NAC concentration (Figure 3). NAC can directly scavenge electrophiles or provide an extracellular source of cysteine to enhance production of GSH.³⁸ On the basis of predicted and measured thiol reactivity for the Br-DBPs, it is likely that NAC acts as a nucleophilic sink, preventing electrophilic damage to the cell. It cannot be determined from this study if NAC or GSH were directly interacting with BAN or BAM or neutralizing ROS generated from some other pathway. However, the differential effect of NAC on the genotoxicity induced by BAN or BAM versus BAA suggests that these electrophilic DBPs target different intracellular nucleophiles (Figure 3).

Role of Ca^{2+} in Br-DBP-Induced Genotoxicity

Disrupted Ca^{2+} homeostasis could contribute to the toxicity of the Br-DBPs investigated in this study. The monoHAAs (including BAA and IAA) strongly inhibited the glycolytic enzyme GAPDH²⁹ and generated oxidative stress through a Ca^{2+} -dependent mechanism.²⁸ In contrast, high concentrations (1 mM) of IAM were required to inhibit GAPDH; in the same study, IAM depleted GSx to a much greater extent than IAA.¹⁸ Interestingly, IAM was used as a model GSH-depleting electrophile and generated Ca^{2+} -dependent oxidative stress.²⁴ CAN also depleted GSH.¹⁶ To investigate the role of increased cytosolic Ca^{2+} in genotoxicity induced by BAA, BAM, or BAN, we treated CHO cells with a constant concentration of each DBP in the presence or absence of the Ca^{2+} -specific intracellular chelator BAPTA-AM. Each of the Br-DBPs (60 μM BAN, 60 μM BAM, or 75 μM BAA) generated genomic DNA damage. The percent tail DNA value, measured with SCGE, generated by each DBP alone was set to 100%, and the effect of BAPTA-AM was measured as the percentage of its positive control. Buffering cytosolic Ca^{2+} significantly ($p < 0.001$) reduced genomic DNA damage induced by each of the Br-DBPs. The highest concentration of BAPTA-AM (200 μM) reduced genomic DNA damage induced by BAA, BAN, or BAM by 76.0, 63.9, and 73.6%, respectively (Figure 4). These data agreed with previous reports that increased cytosolic Ca^{2+} is a critical event in the toxic cascades of IAA^{28,39} and IAM.²⁴ To ensure that the effect of BAPTA-AM on Br-DBP-induced genotoxicity was not a general protective effect, the ROS agent hydrogen peroxide (H_2O_2 , 0.003%, v/v) and the DNA

alkylating agent ethylmethanesulfonate (EMS, 3.8 mM) were evaluated. BAPTA-AM (200 μ M) provided a small (<10%) but significant ($p = 0.001$) protective effect when compared to EMS alone (Figure 4). BAPTA-AM (200 μ M) reduced DNA damage induced by H₂O₂ by approximately 20% ($p = 0.001$) (Figure 4). In comparison to EMS or H₂O₂, BAPTA-AM had a much greater effect on Br-DBP-induced genotoxicity (Figure 4), removing concerns of a non-specific effect.

Genotoxicity of Binary Br-DBP Mixtures

One goal of defining molecular mechanisms is to better understand the toxicity of multiple agents in a complex mixture. Because GSH serves a dual role as an antioxidant and electrophile scavenger,²² we hypothesized that GSH or, in general, the total cellular-free thiol pool functions as a threshold for preventing genomic DNA damage as a result of an insult from soft electrophile(s). Therefore, any DBP or soft electrophile that alkylates thiols reduces the threshold of toxicity for another soft electrophile and, thus, may have a potentiating effect. To test this hypothesis, we investigated the potentiating effect of BAA or BAM on BAN genotoxicity.

BAN alone generated a concentration-dependent increase in DNA damage. BAM alone generated small increases in genomic DNA damage (2.7% tail DNA with 10 μ M and 13.8% tail DNA with 20 μ M). However, the addition of BAM (10 or 20 μ M) expressed a potentiating effect on the genotoxicity of BAN (Figure 5A). For the BAN + BAM mixture, the genotoxicity was not the sum of the genotoxicity derived from each individual chemical but rather the addition of BAM below its toxic threshold (10 μ M) potentiated the toxicity of BAN. The additions of 10 or 20 μ M BAM shifted the BAN concentration–response curve to the left in the manner of a dose additive effect (Figure 5A). For example, the percent tail DNA value (62.3%) induced by 20 μ M BAN + 20 μ M BAM, was significantly greater ($p = 0.001$) than the sum of the two independent exposures (Figure 5A). These data support the hypothesis that the soft electrophiles in this DBP mixture have a cumulative toxicity and support previous findings of dose additivity in S_N2-reactive electrophiles.^{12–14}

Alone, BAA generated small increases in genomic DNA damage (2.15 and 4.15% tail DNA) at 50 and 100 μ M, respectively. The 4.15% tail DNA value was statistically significant ($p = 0.008$) when compared to its concurrent negative control. The addition of BAA to BAN did not generate significant increases in genotoxicity compared to BAN alone at any of the measured concentrations (Figure 5B). Overall, the percent tail DNA values for the BAA + BAN mixtures were similar to the BAN alone concentration response. The data presented in panels A and B of Figure 5 indicated that the addition of BAM but not BAA increased the toxicity of BAN. BAA did not have a potentiating effect on BAN toxicity and, because no additive toxicity was observed, supported the *in silico*, *in chemico*, and *in vitro* observations of differential thiol reactivities and different MIEs for BAA versus BAN and BAM. Furthermore, these data indicated that potentiation of genotoxic responses could be predicted by the *in silico* estimation of E_{LUMO} values. Thus, it is possible that E_{LUMO} estimates could be used to identify DBPs within the same soft electrophile (or thiol-reactive) CMG.

Dose additivity was reported with HANs and ethyl- α -halogenated acetates,¹⁴ compounds structurally similar to the HAAs. However, in those studies, GSH reactivity for ethyl bromoacetate (EBAC)¹³ was higher than BAN and the E_{LUMO} predicted for EBAC (-1.12 eV) was closer to BAN and BAM than BAA (Table 1). The deviation from additivity observed with BAA in the present study (Figure 5B) may stem from differences in thiol reactivity between BAM and EBAC and are reflected in the above-mentioned E_{LUMO} values.

While having a common site of reactivity (Figure 1), BAN and BAM were more thiol-reactive than BAA (panels A and B of Figure 2). The biological consequence of the differential thiol reactivity is that these compounds target different biological macromolecules, generating toxicity with different MIEs. While the initiating events appear different, the toxic cascades of these molecules converge at the point where intracellular Ca^{2+} homeostasis is disrupted (Figure 4). MIEs of toxicity include inhibited sarcoc/endoplasmic reticulum calcium ATPase (SERCA) activity⁴⁰ or unfolded protein-derived endoplasmic reticulum (ER) stress; our evidence indicates that increased cytosolic Ca^{2+} is an important factor in the generation of ROS-mediated genotoxicity for the DBPs analyzed.

We mapped LUMO density and estimated E_{LUMO} to predict sites of reactivity and thiol reactivity for three monobrominated DBPs. The LUMO maps indicated that the site of reactivity for each Br-DBP was the halogenated carbon adjacent to the activated primary alkyl halide. This α -halogenated structure was recently identified as an indicator of GSH reactivity.²¹ The estimated E_{LUMO} value for BAA (4.0 eV) was much higher than those predicted for BAM (-0.66 eV) or BAN (-1.57 eV). The high E_{LUMO} value for BAA was an indicator of low thiol reactivity, both *in chemico* and *in vitro*, whereas the lower E_{LUMO} values for BAN and BAM predicted higher thiol reactivity. Increasing the thiol pool with exogenous NAC reduced the genotoxic damage of BAN or BAM but had little effect with BAA. These data supported the hypothesis that soft electrophiles or low E_{LUMO} electrophiles generate toxicity by depleting the cellular thiol pool. In this study, low E_{LUMO} electrophiles expressed additive toxicity, with BAM potentiating the genotoxic effect of BAN. E_{LUMO} was a useful metric of thiol reactivity and may be used to predict depletion of GSH or total cellular thiols as a MIE. The ability of E_{LUMO} to predict toxicity in more complex molecules needs to be evaluated; however, we suggest that E_{LUMO} could serve as a useful parameter for predicting toxic effects among DBPs in a complex mixture. The significance of this research is that soft electrophile DBPs could be an important CMG. *In silico* estimates of E_{LUMO} could be used to identify the most relevant DBPs that are the forcing factors of the toxicity of finished drinking waters.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

LUMO maps for (A) bromoacetaldehyde, (B) bromoacetate, (C) bromoacetonitrile, and (D) bromoacetamide. The molecular backbone is overlaid. In each case, the bromine atom is set out of plane behind the molecule. The continuum of electron deficiency ranges from blue (high) to red (low); thus, the blue sites are probable sites for nucleophilic attack.

Figure 2.

(A) Effect of Br-DBPs on free thiol levels in chemico. The rank order of increasing thiol reactivity was BAM > BAN $\gg$ BAA. (B) Effect of Br-DBPs on total GSH in CHO cells; the values represent the mean $\pm$ standard error (SE). Each Br-DBP reduced cellular GSH; depletion by BAM and BAN was much greater than BAA.

Figure 3.

Genomic DNA damage in CHO cells by Br-DBPs as a function of NAC. NAC strongly inhibited the genotoxicity induced by 60 μM BAM or 60 μM BAN, with a small effect on 250 μM BAA.

Figure 4.

Effect of the Ca^{2+} -specific chelator BAPTA-AM on genomic DNA damage induced by BAA (75 μM), BAN (60 μM), BAM (60 μM), EMS (3.8 mM), or H_2O_2 (0.003%). Each genotoxin served as its 100% DNA damage concurrent positive control. Chelating Ca^{2+} greatly reduced genomic DNA damage induced by each of the Br-DBPs. In these experiments, 75 μM BAA generated 50.75% tail DNA. The genotoxic effect of BAA diminished in our CHO model over time. BAA genotoxicity was monitored to maintain a suitable percent tail DNA for comparison to BAN and BAM.

Figure 5.

Effect of binary mixtures of Br-DBPs on genotoxic response in CHO cells. (A) Addition of BAM (○ and ▼) enhanced BAN-mediated genotoxicity. (B) Addition of BAA (○ and ▼) had a minimal effect on BAN-induced genotoxicity.

Table 1
 E_{LUMO} and E_{HOMO} Values Estimated for BAA, BAM, and BAN

Br-DBP	E_{LUMO} (eV)	E_{HOMO} (eV)
bromoacetate	4.00	−0.66
bromoacetamide	−0.66	−7.06
bromoacetonitrile	−1.57	−8.33