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Comparative Quantitative Toxicology and QSAR Modeling of the Haloacetonitriles: Forcing Agents of Water Disinfection Byproduct Toxicity

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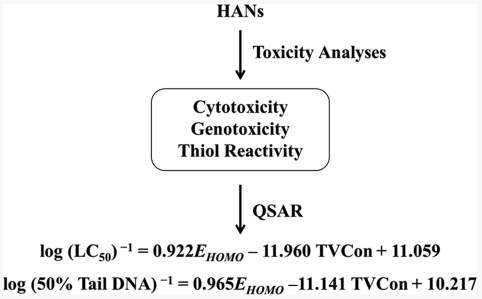
ABSTRACT: The haloacetonitriles (HANs) is an emerging class of nitrogenous-disinfection byproducts (N-DBPs) present in disinfected drinking, recycled, processed wastewaters, and reuse waters. HANs were identified as primary forcing agents that accounted for DBP-associated toxicity. We evaluated the toxic characteristics of iodoacetonitrile (IAN), bromoacetonitrile (BAN), dibromoacetonitrile (DBAN), bromochloroacetonitrile (BCAN), tribromoacetonitrile (TBAN), chloroacetonitrile (CAN), dichloroacetonitrile (DCAN), trichloroacetonitrile (TCAN), bromodichloroacetonitrile (BDCAN), and chlorodibromoacetonitrile (CDBAN). This research generated the first quantitative, comparative analyses on the mammalian cell cytotoxicity, genotoxicity and thiol reactivity of these HANs. The descending rank order for HAN cytotoxicity was TBAN ≈ DBAN > BAN ≈ IAN > BCAN ≈ CDBAN > BDCAN > DCAN ≈ CAN ≈ TCAN. The rank order for genotoxicity was IAN ≈ TBAN ≈ DBAN > BAN > CDBAN ≈ BDCAN ≈ BCAN ≈ CAN ≈ TCAN ≈ DCAN. The rank order for thiol reactivity was TBAN > BDCAN ≈ CDBAN > DBAN > BCAN > BAN ≈ IAN > TCAN. These toxicity metrics were associated with membrane permeability and chemical reactivity. Based on their physiochemical parameters and toxicity metrics, we developed optimized, robust quantitative structure activity relationship (QSAR) models for cytotoxicity and for genotoxicity. These models can predict cytotoxicity and genotoxicity of novel HANs prior to analytical biological evaluation.



INTRODUCTION

Although the disinfection of drinking water was an outstanding public health achievement of the 20th century,[1] an unintended consequence was the generation of toxic disinfection byproducts (DBPs).[2,3] Since their discovery[4,5] over 600 DBPs were characterized,[2,6] a fraction of the total organic halogen in disinfected water.[7] Approximately 100 DBPs have undergone systematic, quantitative, comparative toxicological analyses.[8−10] The U.S. EPA regulates 11 DBPs; none are nitrogen-containing (N-DBPs) or iodinated (I-DBPs).[11] China regulates 14 DBPs in drinking water, including one N-DBP, cyanogen chloride.[12] Yet unregulated N-DBPs and I-DBPs represent the most toxic classes in drinking waters.[9,13−18]

Because of the concentration of haloacetonitriles (HANs) in drinking water and their toxicity, HANs are emerging as major forcing agents in the toxicity of disinfected waters.[7,19−24] The HANs contain a cyano group attached to the α-carbon with halogen substitution; the α-carbon and cyano carbon are reactive centers.[25] The formation and degradation of HANs as metastable DBPs may affect the toxicity of water within a distribution network.[26,27] The World Health Organization guidelines for dichloroacetonitrile (DCAN) and dibromoacetonitrile (DBAN) are 20 μg/L and 70 μg/L, respectively.[28] From the U.S., EPA reports HANs were found up to 41 mg/L.[29] Other HANs, including brominated species, were defined in the U.S. Nationwide Occurrence Study with total HANs at a maximum of 14 mg/L.[7] In the present study the toxic characteristics of 10 HANs were evaluated: iodoacetonitrile (IAN), bromoacetonitrile (BAN), DBAN, bromochloroacetonitrile (BCAN), tribromoacetonitrile (TBAN), chloroacetonitrile (CAN), DCAN, trichloroacetonitrile (TCAN), bromodichloroacetonitrile (BDCAN), and chlorodibromoacetonitrile (CDBAN) (Table 1, Supporting Information (SI) Table S1).

Source waters[30−32] and wastewaters[23] with high bromide and nitrogen concentrations enhanced the formation of N-DBPs. Natural organic matter, pharmaceutical, and personal care products in source waters may lead to N-DBP formation.[33,34] Characteristics of source waters as well as disinfection methods can generate increased levels of N-DBPs.[21,32,35−37] N-DBPs were more toxic than carbonaceous DBPs[9,15] and HANs were forcing agents of measured or calculated toxicity.[19,21−23,35,38−40] These facts indicate the importance of this DBP class in potential health risks.[8,16,41]

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Table 1. Values of Physical−chemical, Quantum-Chemical and Topological Descriptors of 10 Haloacetonitriles[a]

haloacetonitrile, abbreviation, CAS	logP	R	E_{HOMO}(eV)	E_{LUMO}(eV)	μ	ClsC	ShpA	TIndx	BIndx	SDe	SVDe	TVCon	TCon	Windx	S (Å^2)
iodoacetonitrile, IAN, 624−75−9	0.61	24.2	−9.46	−0.584	3.313	4	2.25	76	237	6	11.49	0.227	0.500	10	102.03
bromoacetonitrile, BAN, 590−17−0	0.20	19.0	−10.81	−0.676	3.290	4	2.25	76	237	6	12.00	0.158	0.500	10	96.51
dibromoacetonitrile, DBAN, 3252−43−5	0.47	26.7	−10.83	−1.103	2.626	5	3.20	112	712	8	14.00	0.129	0.408	18	111.42
bromochloroacetonitrile, BCAN, 83463−62−1	0.38	23.8	−11.15	−1.133	2.538	5	3.20	112	712	8	13.78	0.146	0.408	18	107.16
tribromoacetonitrile, TBAN, 75519−19−6	1.48	34.4	−10.96	−1.531	1.963	6	4.17	152	1661	10	16.00	0.112	0.354	28	136.77
chloroacetonitrile, CAN, 107−14−2	0.11	16.1	−11.55	−0.499	3.255	4	2.25	76	237	6	11.78	0.179	0.500	10	92.27
dichloroacetonitrile, DCAN, 3018−12−0	0.29	20.9	−11.83	−0.967	2.421	5	3.20	112	712	8	13.56	0.166	0.408	18	102.94
trichloroacetonitrile, TCAN, 545−06−2	1.21	25.7	−12.12	−1.258	1.342	6	4.17	152	1661	10	15.33	0.163	0.354	28	123.84
bromodichloroacetonitrile, BDCAN, 60523−73−1	1.30	28.6	−11.37	−1.549	1.654	6	4.17	152	1661	10	15.56	0.144	0.354	28	128.12
chlorodibromoacetonitrile, CDBAN, 144772−39−4	1.39	31.5	−11.03	−1.566	1.838	6	4.17	152	1661	10	15.78	0.127	0.354	28	132.44

[a]LogP = octanol−water partition coefficient. R = molar refractivity. E_{HOMO} = energy of the highest occupied molecular orbital. E_{LUMO} = Energy of the lowest unoccupied molecular orbital. μ = dipole moment. ClsC = cluster count. ShpA = shape attribute. TIndx = molecular topological index. BIndx = Balaban index. SDe = sum of degrees. SVDe = sum of valence degrees. TVCon = total valence connectivity. TCon = total connectivity. Windx = Wiener index. S = polar surface area.

The mechanisms of adverse biological effects induced by HANs fall into two general categories, acute direct impacts and delayed cell cycle impacts. Under in vivo exposure, N-DBPs induced adverse effects in zebrafish;[42] these results agreed with Chinese hamster ovary (CHO) cell toxicity.[9] DCAN induced developmental toxicity, reduced hatchability, and increased malformations.[43] A metabolomics study in mice found that HANs increased toxicity with increasing numbers of bromine substituents; these bromo-HANs induced oxidative stress-associated disruptions in amino acid, energy and lipid metabolic processes.[44] Oxidative stress was a significant mechanism of DCAN-induced hepatic mitochondrial injury in rats.[45] Toxicity pathway-based studies uncovered a potential to damage or inhibit proteins and enzymes. A soft electrophilic mechanism of action was suggested.[10,46] When analyzed with antioxidant response element (ARE)-driven transcription of a reporter gene, the HANs were among the most potent in inducing oxidative stress in human cells. In addition BAN induced altered expression for genes related to inflammation and immune responses.[47] The HANs induced p53 activation which is used as a marker for mammalian genotoxicity and carcinogenicity.[10] Quantitative structure−activity relationship (QSAR) modeling suggested that the genotoxicity of DCAN, DBAN, CAN, and IAN may be related to their molecular topological properties.[48]

HANs expressed aberrant impacts on the cell cycle.[49,50] Exposure of IAN, BAN, or CAN to CHO cells, at noncytotoxic concentrations, followed by the release from HAN treatment resulted in the accumulation of hyperploid (8N) cells over time. The potency for cell cycle alteration followed the rank order of IAN > BAN ≫ CAN. Proliferation of HAN-treated cells was suppressed for as long as 52 h. Enlarged cell size was observed without cytotoxicity with HAN treatment-induced mitosis override. This cell cycle M-phase blockage may involve the inhibition of nuclear topoisomerases.[51] Cells with multiple genomes would result in aneuploidy since extra centrosomes could compromise the assembly of bipolar spindles.[49,51] In yeast cells, DBAN delayed the transition from G1 to S phase in the cell cycle and blocked checkpoint kinase 1 (Chk1) at compromised DNA replication forks.[50] HANs may adversely impact a process or a protein that is necessary at the beginning of S phase which is also required at damaged DNA replication forks. Genomic DNA damage, the induction of aneuploidy and DNA replication stress are associated with cancer progression.[52,53] Specific HANs are carcinogenic in rats and mice.[54,55]

With increased concern of HANs in drinking water and as forcing agents for toxicity, we expanded the comparative CHO cell toxicity database. The objectives of this research included, (i) to generate the first quantitative, comparative analyses on the mammalian cell cytotoxicity, genotoxicity and thiol reactivity of 10 HANs, (ii) to determine the rank order of their cytotoxicity, genotoxicity, and thiol reactivity based on statistical analyses, and (iii) to determine an association among selected physicochemical characteristics based on correlation analyses and QSAR modeling of HANs and toxicity metrics.

MATERIALS AND METHODS

Haloacetonitriles. The sources, purity and physicochemical characteristics of the HANs are presented in Table 1 and SI Table S1.

Biological and Chemical Reagents, CHO Cells. For the in vitro cytotoxicity and genotoxicity experiments CHO K1 cell line (AS52, clone 11−4−8) was employed. These CHO

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Table 2. CHO Cell Cytotoxicity of the Haloacetonitrile DBPs

HAN	lowest cytotoxic conc. (μM)[a]	mean LC_{50} value (μM ± SE)[b]	r^2[c]	ANOVA test statistic[d]	mean CTI value ± SE[e]
IAN	0.1	3.27 ± 0.05	0.98	$F_{12, 163}$ = 148.4; $P \leq 0.001$	307.28 ± 4.41
BAN	1.0	3.10 ± 0.06	0.98	$F_{11, 228}$ = 98.3; $P \leq 0.001$	325.83 ± 7.05
DBAN	1.0	2.79 ± 0.09	0.99	$F_{11, 179}$ = 271.5; $P \leq 0.001$	364.57 ± 11.98
BCAN	7.0	8.20 ± 0.51	0.96	$F_{11, 171}$ = 36.2; $P \leq 0.001$	130.84 ± 8.24
TBAN	1.0	2.71 ± 0.04	0.99	$F_{11, 100}$ = 401.8; $P \leq 0.001$	369.56 ± 5.37
CAN	50.0	66.09 ± 1.63	0.99	$F_{13, 188}$ = 65.9; $P \leq 0.001$	15.3 ± 0.38
DCAN	10.0	55.03 ± 3.23	0.99	$F_{10, 171}$ = 63.4; $P \leq 0.001$	19.48 ± 1.29
TCAN	25.0	158.55 ± 6.01	0.93	$F_{17, 282}$ = 36.8; $P \leq 0.001$	6.55 ± 0.25
BDCAN	6.0	10.22 ± 0.12	0.97	$F_{15, 131}$ = 141.1; $P \leq 0.001$	98.10 ± 1.12
CDBAN	6.0	8.14 ± 0.18	0.98	$F_{11, 79}$ = 78.5; $P \leq 0.001$	123.59 ± 3.22

[a]The lowest HAN concentration that induced a statistically significant reduction as compared to the negative controls. [b]LC_{50} is the HAN concentration that induced a cell density of 50% of the negative controls. The mean and the standard error (SE) were derived using bootstrap statistics. [c]The r^2 is the coefficient of determination for the regression analysis of the concentration−response data. [d]The ANOVA degrees of freedom and the resulting probability value. [e]CTI = $LC_{50}^{-1} \times 10^3$.

cells are genomically stable, adherent, have normal morphology, express cell contact inhibition and grow as a monolayer without expression of neoplastic foci.[56,57] A description of the growth conditions for the CHO cells is in the SI.

CHO Cell Chronic Cytotoxicity Analyses. CHO cell chronic cytotoxicity captures adverse biological impacts that result in the reduction in cell density after exposure to each HAN for 3 days.[9] In this study the cytotoxicity of TBAN, BDCAN, and CDBAN was analyzed; data for the other HANs were previously published.[24] Detailed procedures for this assay were published.[9,58]

Single Cell Gel Electrophoresis Genotoxicity Analyses. Single cell gel electrophoresis (SCGE or comet) analytically determines genomic DNA damage including DNA single- and double-strand breaks, incomplete excision repair sites, and alkali-labile sites in nuclei.[59−61] The SCGE metric was the average %Tail DNA value and the 50%Tail DNA value was calculated after regression analyses. The details of SCGE analyses are presented in the SI and were published.[9]

***N*-Acetylcysteine Thiol Reactivity Analyses.** The *N*-acetylcysteine (NAC) thiol reactivity screen was developed to identify potential adverse biological effects induced by toxic agents.[46,62−64] HANs were reacted with NAC and the response was recorded spectroscopically. The details for these HAN thiol reactivity analyses are presented in the SI and the procedure was previously published.[62]

Statistical Analyses for the Analytical Biology. Using a one-way analysis of variance (ANOVA) a test for significance was conducted. If a significant F value of $P \leq 0.05$ was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted with the power $(1-\beta) \geq 0.8$ at $\alpha = 0.05$.[65,66] LC_{50} values, 50%Tail DNA values and EC_{50} values were determined for cytotoxicity, genotoxicity, and NAC-thiol reactivity, respectively. The mean toxicity index values (±SE) were calculated employing bootstrap statistics[67,68] The definitions for the index values are defined in the SI and in Tables 2−4. The Pearson product-moment correlation test analyzed functional associations among HAN groups and biological and physicochemical metrics.[14,69] HAN concentration−response curves for cytotoxicity (SI Figures S1−S10), genotoxicity (SI Figures S11−S20), and thiol reactivity (SI Figures S21−S30) with corresponding statistical analyses (SI Tables S2−S27) are presented in the SI.

Development of QSAR Model. By using stepwise multiple linear regression (MLR),[70] two QSAR models were developed for the CHO assays based on the LC_{50} values for cytotoxicity and the 50%Tail DNA values for genotoxicity. For the descriptors, the log$P$ values were estimated using the KOWWIN program (v. 1.69). The values of the energy of the highest occupied molecular orbital (E_{HOMO}), the energy of the lowest unoccupied molecular orbital (E_{LUMO}) and dipole moment (μ) were calculated with MOPAC2016 using the PM7 method. The values of molar refractivity (R) and molar surface area (S) were generated from Percepta Platform software (ACD Laboratories) and ChemAxon, respectively. Other descriptors (Table 1) were calculated using ChemOffice 2010 (Cambridge Soft). By performing the stepwise elimination of independent variables with the SPSS 22.0 software, appropriate variables were screened and selected from 15 candidate descriptors.

Statistical Metrics for Validation of the Developed QSAR Models. Several statistical parameters acquired from the regression equations including the significance level (P), the determination coefficient (R^2), variance ratio (F), the root-mean-square error (RMSE), and mean absolute error (MAE) were used to evaluate the prediction error. The established QSAR models were validated by using cross-validation through the leave-one-out (LOO) procedure to confirm their preferable prediction performance and practicability.[71] Y-scrambling validation was applied to check the chance correlation of the QSAR models.[72] For each developed QSAR equation, values of R^2 and Q^2_{LOO} were obtained from 50 randomly generated QSAR models, which should be lower than those of the developed model. The collinearity among the modeling variables needed to be reduced by evaluating the variance inflation factor (VIF).[73] The applicability domains (ADs) of the developed models were assessed using Williams plots of standardized residuals (δ) versus leverage (h) which expressed simple and straightforward graphical visualization of outliers. The leverage threshold (h^*) was calculated as $h^* = \frac{3k}{n}$, where k is the number of predictors plus one and n was the number of the tested compounds.[74]

RESULTS AND DISCUSSION

The HANs and other N-DBPs accounted for the majority of toxicity associated with disinfected waters; HANs are more cytotoxic and genotoxic than regulated DBPs.[9,15,18,22,24] With the increased demand for more quantitative comparative

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toxicity data we expanded the number of HANs analyzed with additional toxicity end points plus QSAR modeling.

Comparative CHO Cell Chronic Cytotoxicity. TBAN, BDCAN, and CDBAN were compared with other HANs (Figure 1, Table 2). The individual cytotoxicity concentration−response curves for 10 HANs are presented in SI Figures S1−S10 and the ANOVA test of the cytotoxicity data of TBAN, BDCAN and CDBAN are presented in SI Tables S2−S4. These trihalo-HANs were highly toxic with mean LC_{50} values for TBAN, BDCAN, and CDBAN of 2.71, 10.22, and 8.14 μM, respectively (Table 2). Using cytotoxic index (CTI) values the statistical rank order from most toxic to least toxic was TBAN ≈ DBAN > BAN ≈ IAN > BCAN ≈ CDBAN > BDCAN > DCAN ≈ CAN ≈ TCAN (Table 2). Applying an ANOVA test (Holm-Sidak all pairwise multiple comparison ($F_{9,175}$ = 744.8; P < 0.001) SI Table S5) of the CTI values, the HANs separated by > were significantly different while those separated by ≈ were not.

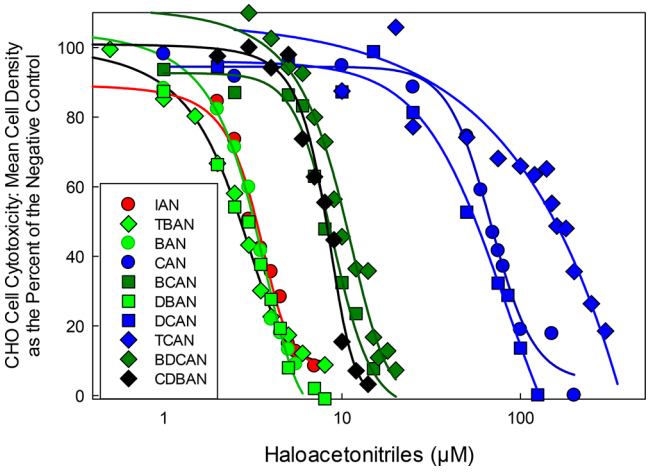

Figure 1. CHO cell chronic cytotoxicity concentration−response curves of the haloacetonitriles. SI Figures S1−S10 present each HAN cytotoxicity concentration−response curve with error bars. The ANOVA statistical analysis for TBAN, BDCAN, and CDBAN cytotoxicity are presented in SI Tables S2−S4.

Comparative CHO Cell Genotoxicity. We published the SCGE genomic DNA damage analyses of seven HANs with the tail moment as the biological metric.[24] However, the current preferred metric for SCGE is the %Tail DNA value.[59,75] Using % Tail DNA values as the genotoxicity metric, a comparison of the SCGE concentration−response curves are presented in Figure 2 (individual concentration−response curves are presented in SI Figures S11−S20). Table 3 presents the statistical analyses of the genotoxicity of 10 HANs including the lowest concentration that induced a significant genotoxic response as well as the 50% Tail DNA values (SI Tables S6−S15). Using genotoxic index (GTI) values the statistical rank order from most genotoxic to least genotoxic was IAN ≈ TBAN ≈ DBAN > BAN > CDBAN ≈ BDCAN ≈ BCAN ≈ CAN ≈ TCAN ≈ DCAN (where CDBAN > BCAN, CAN, TCAN, DCAN, and BDCAN > CAN, TCAN, DCAN) (Table 3). Applying an ANOVA test (Holm-Sidak all pairwise multiple comparison ($F_{9,104}$ = 279.9; P < 0.001) SI Table S16 of the GTI values, those HANs separated by > were significantly different while those separated by ≈ were not.

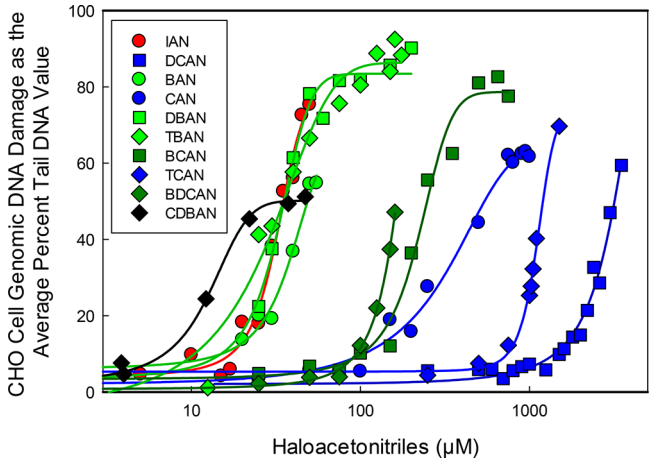

Figure 2. CHO cell genotoxicity, as the %Tail DNA, concentration−response curves of the haloacetonitriles. SI Figures S11−S20 present each HAN genotoxicity concentration−response curve with error bars, acute cytotoxicity and its ANOVA statistical analysis (SI Tables S6−S15) are presented in the SI.

Comparative NAC Thiol Reactivity. The NAC thiol reactivity was assessed; the comparative concentration−response curves are presented in Figure 3 (SI Figures S21−S30). A statistical analyses of the thiol reactivity including the lowest concentration that induced a significant response as well as their EC_{50} values is presented in Table 4 (SI Tables S17−S26). The rank order from most thiol reactive to least thiol reactive using the thiol reactivity index (TRI) values was TBAN > BDCAN ≈ CDBAN > DBAN > BCAN > BAN ≈ IAN > TCAN (Table 4). Applying an ANOVA test of the TRI values (Holm-Sidak all pairwise multiple comparison ($F_{7,53}$ = 809.6; P < 0.001) (SI Table S27), those HANs separated by > were significantly different while those separated by ≈ were not. CAN and DCAN did not express NAC thiol reactivity.

QSAR Models. Table 1 lists the calculated values of the candidate descriptors. To avoid chance correlation, the ratio of compound number to variable number in models should be >5:1. Thus, only two descriptors were involved based on 10 HANs. Since CAN and DCAN did not express a thiol-reactivity response the corresponding QSAR equation was not developed. To select the most appropriate descriptors and to develop the QSAR models, stepwise multiple regression based on the Elimination Selection Stepwise Regression (ES-SWR) algorithm was performed using SPSS 22.0. The optimum QSAR models for cytotoxicity and genotoxicity are shown in eqs 1 and 2, respectively To check model predictability and robustness, model validation was conducted and both models expressed high goodness-of-fit (Figures 4 and 5); detail criteria and explanation for validation tests are listed in the SI.

$$\log(LC_{50})^{-1} = 0.922E_{HOMO} - 11.960TVCon + 11.059 \quad (1)$$

n = 10, R^2 = 0.933, Q^2_{LOO} = 0.617, R^2_{YS} = 0.230, Q^2_{YS} = −0.678, RMSE = 0.190, MAE = 0.128, F = 48.6, P < 0.0001, VIF = 1.19

$$\log(50\%\text{Tail DNA})^{-1} = 0.965E_{HOMO} - 11.141TVCon + 10.217 \quad (2)$$

n = 10, R^2 = 0.887, Q^2_{LOO} = 0.714, R^2_{YS} = 0.221, Q^2_{YS} = −0.465, RMSE = 0.261, MAE = 0.209, F = 27.6, P < 0.001, VIF = 1.19 where n is the number of compounds in the data

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Table 3. CHO Cell SCGE %Tail DNA Genotoxicity Analyses of the Haloacetonitrile DBPs

HAN	lowest genotoxic conc. (μM)[a]	mean 50%Tail DNA (μM ± SE)[b]	r^2[c]	ANOVA test statistic[d]	mean GTI value ± SE[e]
IAN	25	34.24 ± 0.76	0.98	$F_{11,56} = 57.72$; $P \leq 0.001$	29.35 ± 0.63
BAN	20	48.19 ± 1.06	0.98	$F_{6,36} = 68.42$; $P \leq 0.001$	20.9 ± 0.47
DBAN	25	35.50 ± 0.37	0.98	$F_{9,40} = 182.3$; $P \leq 0.001$	28.21 ± 0.29
BCAN	200	250.1 ± 12.3	0.98	$F_{11,44} = 53.46$; $P \leq 0.001$	4.11 ± 0.23
TBAN	25	37.76 ± 3.23	0.97	$F_{11,51} = 19.60$; $P \leq 0.001$	28.37 ± 2.28
CAN	250	517.9 ± 26.2	0.98	$F_{13,44} = 46.49$; $P \leq 0.001$	2.00 ± 0.13
DCAN	2200	3083 ± 85.4	0.98	$F_{17,62} = 19.20$; $P \leq 0.001$	0.33 ± 0.01
TCAN	750	1187 ± 15.2	0.98	$F_{8,33} = 160.4$; $P \leq 0.001$	0.84 ± 0.01
BDCAN	125	163.29 ± 1.45	0.99	$F_{7,29} = 16.25$; $P \leq 0.001$	6.13 ± 0.05
CDBAN	100	139.73 ± 2.89	0.83	$F_{8,41} = 26.81$; $P \leq 0.001$	7.19 ± 0.15

[a]The lowest HAN concentration that induced a statistically significant increase in the electrophoretic migration of genomic DNA from the nucleus as compared to the negative control. [b]The HAN concentration that induced a DNA migration from the nuclei of 50%. The mean and the standard error (SE) were derived using bootstrap statistics. [c]The r^2 is the coefficient of determination. [d]The ANOVA degrees of freedom and the resulting probability value. [e]GTI = 50% Tail DNA^{-1} × 10^3.

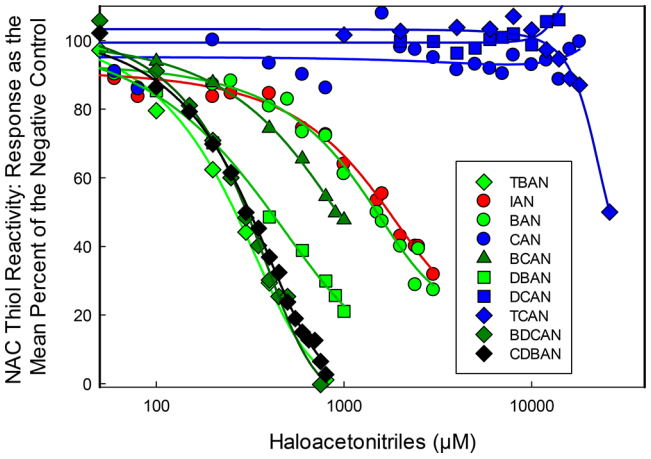

Figure 3. NAC thiol reactivity concentration−response curves of the haloacetonitriles. SI Figures S21−S30 present each HAN NAC thiol reactivity concentration−response curve with error bars and its ANOVA statistical analysis (SI Tables S17−S26) are presented in the SI.

set; R^2 is the determination coefficient; Q^2_{LOO} is the leave-one-out cross-validation coefficient; R^2_{YS} and Q^2_{YS} are Y-scrambling technique parameters; RMSE and MAE are the root-mean-square error and the mean absolute error for the data set, respectively; P is the significance level.

Two descriptors E_{HOMO} and TVCon are involved in the optimum QSAR models. These two descriptors may reveal the toxicity mechanisms of HANs to some extent. Many chemical reactions are inextricably linked to the frontier molecular orbitals of reacting species.[76,77] E_{HOMO} is related to the relative nucleophilicity; a higher value indicates the corresponding compound may possess higher electron donating ability, thus having a higher potential to react with electrophiles.[78,79] Various adverse biological outcomes can result from the electrophilic-nucleophilic interactions via different mechanisms (such as Michael addition, Schiff's base formation, and nucleophilic substitution); however, the reactions are not specific.[80] It was reported that the metabolism of diethylstilbestrol (a carcinogenic synthetic estrogen) generates peroxides, which may react with the electrophilic sites in nucleic acids and lipids.[81] HANs may attack electrophilic sites in biomacromolecules within cells via electrophilic-nucleophilic interactions, leading to cytotoxicity and genotoxicity. Iodine/bromine-containing HANs have higher toxicity than their chlorinated analogues and express higher E_{HOMO} values. Previous studies reported that cytotoxicity and developmental toxicity of aromatic DBPs correlated well with E_{HOMO}.[69,77] Cytotoxicity induced by halobenzenes were highly related with E_{HOMO} and oxidation may a toxicity mechanism.[82] The

Table 4. NAC Thiol Reactivity Analyses of the Haloacetonitrile DBPs

HAN	lowest NAC response (mM)[a]	EC_{50} value (mM ± SE)[b]	r^2[c]	ANOVA test statistic[d]	mean TRI value ± SE[e]
IAN	0.020	1.71 ± 0.07	0.97	$F_{18,76} = 59.68$; $P \leq 0.001$	0.589 ± 0.026
BAN	0.060	1.50 ± 0.02	0.97	$F_{18,34} = 67.71$; $P \leq 0.001$	0.666 ± 0.008
DBAN	0.100	0.404 ± 0.004	0.99	$F_{7,17} = 505.4$; $P \leq 0.001$	2.48 ± 0.02
BCAN	0.100	0.913 ± 0.01	0.99	$F_{7,17} = 435.1$; $P \leq 0.001$	1.10 ± 0.01
TBAN	0.100	0.263 ± 0.007	0.99	$F_{8,39} = 433.5$; $P \leq 0.001$	3.82 ± 0.09
CAN	NA[f]	NA		NS[g]	NS
DCAN	NA	NA		NS	NS
TCAN	18.0	26.15 ± 0.01*	0.86	$F_{10,22} = 3.698$; $P \leq 0.001$	0.0382 ± 0.0001
BDCAN	0.100	0.302 ± 0.002	0.98	$F_{12,37} = 239.3$; $P \leq 0.001$	3.31 ± 0.02
CDBAN	0.100	0.314 ± 0.006	0.99	$F_{17,63} = 270.0$; $P \leq 0.001$	3.19 ± 0.055

[a]The lowest HAN concentration that induced a statistically significant reduction as compared to the negative control. [b]EC_{50} value is the HAN concentration that induced a reduction in NAC thiol concentration by 50%. The * denotes that the EC_{50} value was generated by extrapolation. The mean and the standard error (SE) were derived using bootstrap statistics. [c]The r^2 is the coefficient of determination for the regression analysis of the concentration−response data. [d]ANOVA degrees of freedom and the resulting probability value. [e]TRI = EC_{50}^{-1} × 10^3. [f]NA = not applicable. [g]NS = not significant.

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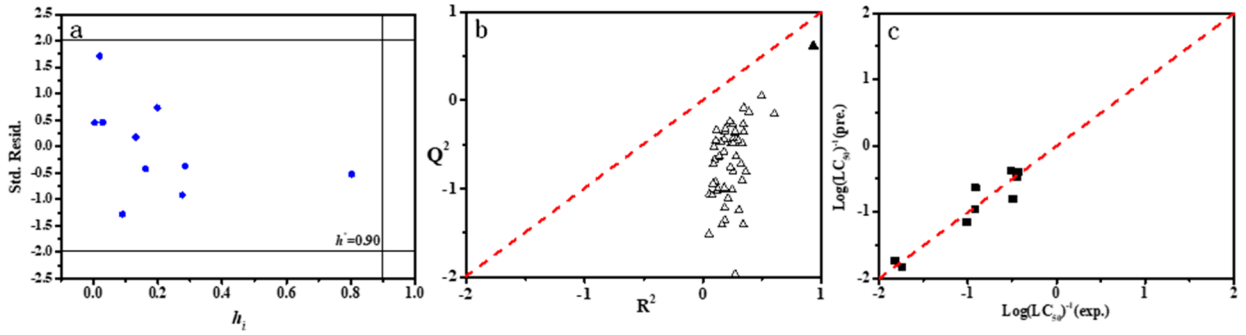

Figure 4. (a) Applicability domain of the developed QSAR model for cytotoxicity, (b) Scatter plot of the recorded Q^2_{YS} and R^2_{YS} for the haloacetonitriles (the filled triangle and empty triangles correspond to the developed QSAR model and random models, respectively), (c) Relationship between the experimental and predicted cytotoxicity data (the red dash straight line is the 45-degree benchmark line).

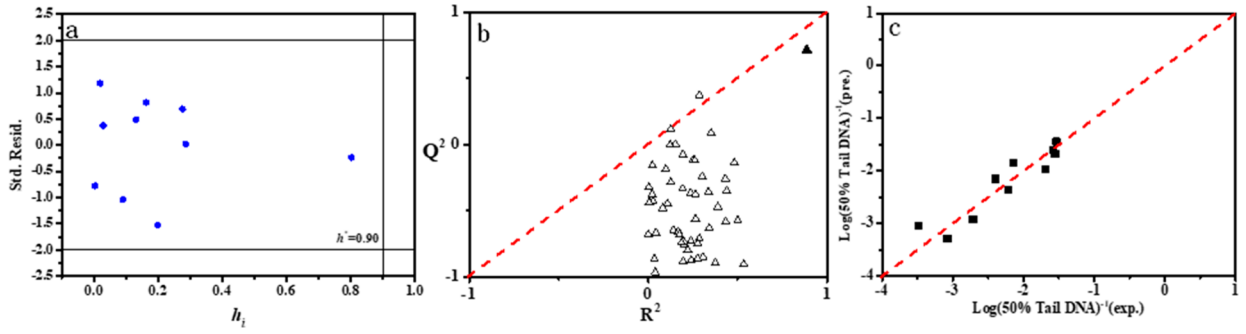

Figure 5. (a) Applicability domain of the developed QSAR model for genotoxicity, (b) Scatter plot of the recorded Q^2_{YS} and R^2_{YS} for the haloacetonitriles (the filled triangle and empty triangles correspond to the developed QSAR model and random models, respectively), (c) Relationship between the experimental and predicted genotoxicity data (the red dash straight line is the 45-degree benchmark line).

genotoxicity of DCAN, DBAN, CAN, and IAN may be related to their molecular topological properties.[48] The two HAN QSAR models in our study confirm the importance of topological properties, not only for genotoxicity, but also for cytotoxicity. Among seven topological parameters, TVCon correlated with cytotoxicity and genotoxicity. The index encodes structural characteristics, e.g., molecular size, degree of branching, shape, unsaturation, heteroatom content, and cyclicity.[83]

These QSAR models may predict cytotoxicity and genotoxicity of novel HANs, especially iodinated HANs. If new iodinated HANs are detected in disinfected waters, toxicity data predicted by these two models would prioritize those HANs to be synthesized for quantitative toxicity. The predicted LC_{50} and 50% Tail DNA values of the recently discovered chloroiodoacetonitrile and other HAN that have not been identified in water are presented in Table 5.[84]

Toxicity Correlations. For the first time a quantitative biological analyses of 10 HANs were compared using the same biological platform. The importance of the halogen atom(s) bound to the α-carbon on toxicity was calculated using index values; the iodinated and brominated HANs were approximately 18× more cytotoxic and 16× more genotoxic than their chlorinated analogues (Tables 2 and 3). The CTI and GTI values were highly and significantly correlated ($r = 0.97$; $P \leq 0.001$).

We conducted multiple correlation analyses using the CTI, GTI, and TRI values with the physicochemical parameters (Table 1). In terms of biological activity, the parameters that demonstrated a correlation with toxicity end points may be broadly divided into membrane permeability (log*P*, S, R) and chemical reactivity (E_{HOMO}, E_{LUMO}, TVCon, SVDe) (Table 1). CTI and GTI values were significantly correlated with E_{HOMO} ($r = 0.73$; $P \leq 0.02$ and $r = 0.79$; $P \leq 0.007$, respectively). NAC thiol reactivity (TRI values) was weakly correlated with log*P* ($r = 0.60$) and highly correlated with R ($r = 0.83$; $P \leq 0.01$). TRI was significantly correlated with SVDe ($r = 0.66$; $P \leq 0.05$), TVCon ($r = -0.71$; $P \leq 0.05$), S ($r = 0.72$; $P \leq 0.05$) ,and E_{LUMO} ($r = -0.75$; $P \leq 0.04$).

Table 5. List of Haloacetonitriles and Their QSAR Model-Predicted Cytotoxicity (LC_{50}) and Genotoxicity (50% Tail DNA) Values

haloacetonitrile	E_{HOMO}(eV)	TVCon	predicted LC_{50} (μM)	predicted 50% TDNA (μM)
diiodoacetonitrile	−9.447	0.266	6.76	72.53
triiodoacetonitrile	−9.461	0.330	41.13	391.30
bromoiodoacetonitrile	−9.632	0.185	1.09	13.86
chloroiodoacetonitrile	−9.708	0.210	2.53	30.99
bromodiiodoacetonitrile	−9.538	0.230	3.07	35.61
chlorodiiodoacetonitrile	−9.588	0.261	7.99	87.74
dibromoiodoacetonitrile	−9.741	0.160	0.69	9.34
dichloroiodoacetonitrile	−9.892	0.206	3.37	42.27

Cytotoxicity, genotoxicity, and thiol reactivity were all highly and significantly correlated with the relative alkylation potency of the HANs ($r = 0.99$; $P \leq 0.002$, $r = 0.97$; $P \leq 0.01$, and $r = 0.99$; $P \leq 0.06$, respectively). The associations with toxicity, alkylation potential[85] and thiol reactivity suggest that the interaction of HANs with biological molecules, especially proteins, may play a part in their overall toxicity.

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The HANs are associated with the induction of reactive oxygen species and oxidative stress. In a study on DNA damage pathway analyses, HAN-mediated interference at the DNA replication fork was reported. Based on ARE activation, the HANs were among the most potent DBPs tested for the induction of oxidative stress in human cells.[10,47] HANs induced p53 activation which is an indicator for mammalian genotoxicity and carcinogenicity.[10] The activation of DNA damage and repair pathways suggested that the genotoxicity of the HANs were structure-dependent, reflected oxidative damage to DNA and were related to their topological properties.[48] Cytotoxicity and genotoxicity are relatively immediate toxic responses. In addition HANs exhibit a delayed toxic response in that they interfere with transcription elements and/or enzymes involved in cell division. The capacity of HANs to react with biological thiols (Table 4) may not only reduce cellular defenses against oxidative stress; direct damage to proteins may be involved. HAN-mediated cell cycle M-phase blockage, the possible inhibition of associated nuclear topoisomerases, HAN effects on the G1 to S phase in the cell cycle, and blocked Chk1 checkpoint kinase strongly imply a direct adverse impact on cellular proteins involved with genomic stability.[49−51]

When disinfected waters were analyzed for DBP levels and for calculated or measured cytotoxicity or genotoxicity, the N-DBPs, and not the regulated DBPs, were found to be the primary forcing agents driving toxicity.[19,21−23,40] In 9 of 11 European drinking waters the primary forcing agents for both cytotoxicity and genotoxicity were N-DBPs; of these the HANs accounted for approximately 70%.[86,87] Attention is being focused on the HANs because of their impact on the toxicity and possible chronic health effects of disinfected water. Information continues to accrue that challenges the primacy of regulated DBPs as health risks as compared to the N-DBPs.[8,22,88] The HANs will play a central role in future evaluations of the risks to the environment and the public health posed by disinfection byproducts.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c02035.

QSAR validation, methods for statistical analyses for the analytical biology, biological and chemical reagents, CHO cells, CHO cell chronic cytotoxicity analyses, detailed concentration−response curves for CHO cell chronic cytotoxicity, acute SCGE genotoxicity, and response curves for the NAC thiol reactivity analyses, statistical analyses (PDF)

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Notes

The authors declare no competing financial interest.

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Comparative quantitative toxicology and QSAR modeling of the haloacetonitriles: forcing agents of water disinfection by-product toxicity

Supporting Information

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Contains 47 pages, 27 tables, and 30 figures

QSAR model validation

According to the acceptable criteria reported previously ($R^2 > 0.700$, $Q^2_{LOO} > 0.600$, $p < 0.05$),[1] the obtained R^2, Q^2_{LOO} and p values indicate the two models with high goodness-of-fit and robustness. The *RMSE* and *MAE* values for both models were relatively small. The *F* values were relatively greater in certain degrees of freedom. The applicability domains which indicated the areas of reliable predictions of the models were characterized using the Williams plot.[1] The HANs in the two data sets are in the corresponding domains, indicating that both the cytotoxicity and genotoxicity data sets have great representativeness (Figures 4a and 5a). According to the Y-scrambling test criteria, the obtained random models have significantly lower prediction accuracies than the two developed models based on experimental data, indicating no accidental correlation in the QSAR models (Figures 4b and 5b).[2] The variance inflation factor (*VIF*) of the two variables is lower than 10, indicating that there is no serious multi-collinearity among the variables and the established models are stable and acceptable. The plot of observed versus predicted log $(LC_{50})^{-1}$ values is shown in Figure 4c, further demonstrating that the LC_{50} values predicted from the developed cytotoxicity model are generally coincident with the observed values. Also, the predicted 50% Tail DNA values generally coincide with the observed values (Figure 5c).

Table S1. Haloacetonitrile source and purity			
Haloacetonitrile & Abbreviation	Formula & CAS	Molecular Weight	Source & Purity
Iodoacetonitrile IAN	C_2H_2IN 624-75-9	166.95	Sigma Aldrich 98%
Bromoacetonitrile BAN	C_2H_2BrN 590-17-0	119.95	Chem Service 97%
Dibromoacetonitrile DBAN	C_2HBr_2N 3252-43-5	198.84	Chem Service 97%
Bromochloroacetonitrile BCAN	$C_2HBrClN$ 83463-62-1	154.39	Chem Service Tech
Tribromoacetonitrile TBAN	C_2Br_3N 75519-19-6	277.74	Cansyn Chem Corp. >90%
Chloroacetonitrile CAN	C_2H_2ClN 107-14-2	75.497	Chem Service 99.5%
Dichloroacetonitrile DCAN	C_2HCl_2N 3018-12-0	109.94	Chem Service 99.5%
Trichloroacetonitrile TCAN	C_2Cl_3N 545-06-2	144.39	Sigma Aldrich 98%
Bromodichloroacetonitrile BDCAN	C_2BrCl_2N 60523-73-1	188.84	Toronto Res. Chem. 98%
Chlorodibromoacetonitrile CDBAN	C_2Br_2ClN 144772-39-4	233.29	Cansyn Chem Corp. >88%

Methods for statistical analyses for the analytical biology

Statistical analyses were conducted for each toxicological assay. After a concentration-response curve from combined replicate experiments (>3) was generated, a test for significance using a one-way analysis of variance (ANOVA) test was conducted. If a significant F value of $P \leq 0.05$ was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted with the power $(1-\beta) \geq 0.8$ at $\alpha = 0.05$ to identify the lowest concentration that was

significantly different from the negative control.[3, 4] After regression analyses, LC_{50} values were determined for CHO cell cytotoxicity, 50%Tail DNA values for CHO cell genotoxicity, and EC_{50} values for NAC-thiol reactivity. Bootstrap statistics were conducted for each assay dataset [5, 6] and mean toxicity index values (±SE) were calculated. We used index values (expressed as μM) such that the larger the value, the more toxic or reactive the sample. The cytotoxicity index (CTI) value is the $LC_{50}^{-1}\times10^3$; the genotoxicity index (GTI) value is the 50%Tail $DNA^{-1}\times10^3$; the thiol reactivity index (TRI) value is the $EC_{50}^{-1}\times10^3$. Using these index values, an ANOVA test was conducted to identify significant differences among specific groups. The Pearson product-moment correlation test analyzed functional associations amongst HAN groups and biological and physicochemical metrics.[7, 8]

Biological and chemical reagents, CHO cells

For the in vitro cytotoxicity and genotoxicity experiments CHO K1 cell line (AS52, clone 11-4-8) was employed.[9, 10] Cells were grown in Hams F12 medium containing 5% fetal bovine serum (FBS), 1% L-glutamine, and 1% antibiotics (0.25 μg/mL amphotericin B, 100 μg/mL streptomycin sulfate, and 100 units/mL sodium penicillin G in 0.85% saline) at 37 °C in a mammalian cell incubator with a humidified atmosphere of 5% CO_2.

CHO cell chronic cytotoxicity analyses

CHO cell cytotoxicity was measured as the reduction in cell density after exposure of CHO cells to each HAN for 72 h compared to untreated concurrent negative controls.[11] Cytotoxicity uncovers a wide array of toxic insults and adverse biological impacts. In this study the

cytotoxicity of TBAN, BDCAN and CDBAN was analyzed; data for the other HANs were previously published.[12] Detailed procedures for this assay were published.[11, 13]

The concentration-response graphs illustrating the CHO cell chronic cytotoxicity of the haloacetonitriles (HANs) are presented in Figures S1 to S10.

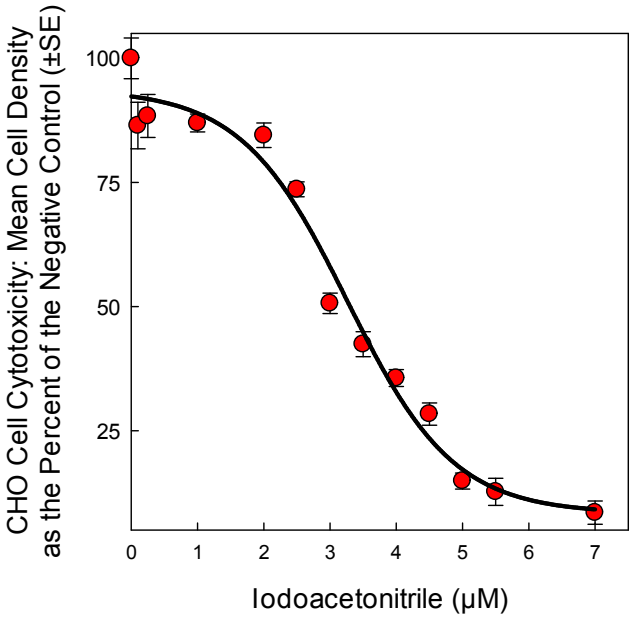

Figure S1. CHO cell cytotoxicity concentration-response curve for IAN. Mean (±SE) LC_{50} value was 3.27 ± 0.05 µM.

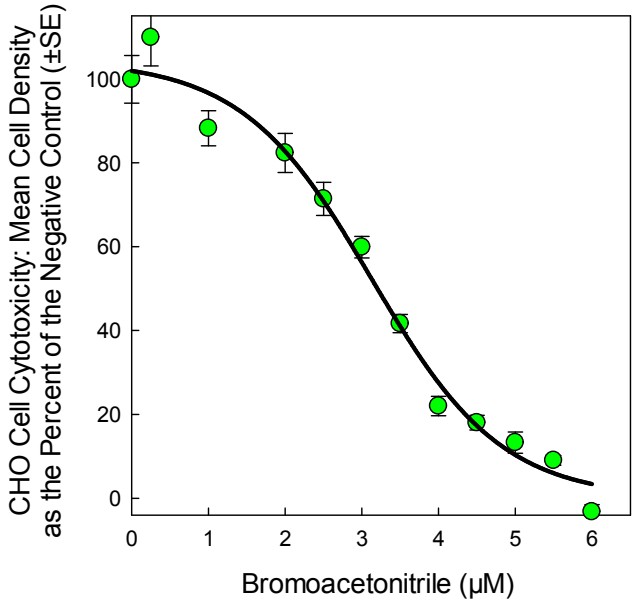

Figure S2. CHO cell cytotoxicity concentration-response curve for BAN. Mean (±SE) LC_{50} value was 3.10 ± 0.06 µM.

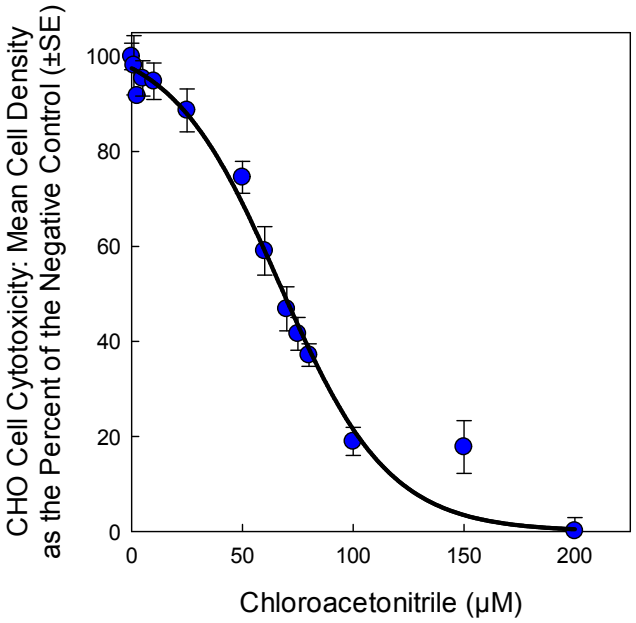

Figure S3. CHO cell cytotoxicity concentration-response curve for CAN. Mean (±SE) LC_{50} value was 66.09 ± 1.63 µM.

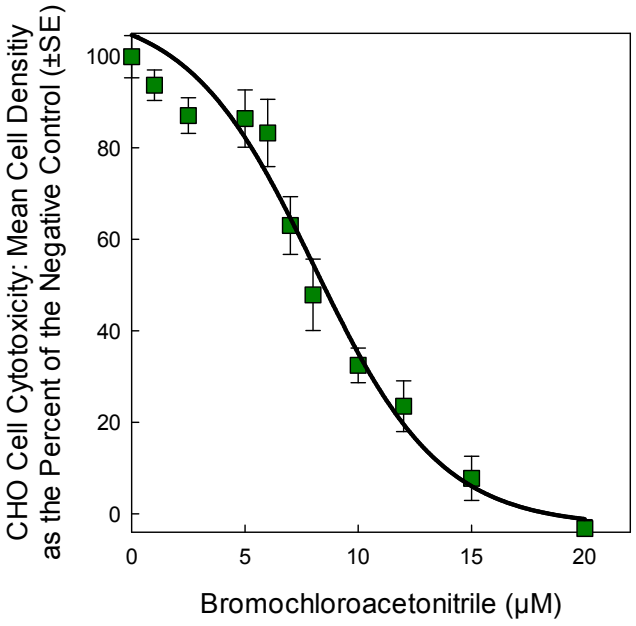

Figure S4. CHO cell cytotoxicity concentration-response curve for BCAN. Mean (±SE) LC_{50} value was 8.20 ± 0.51 µM.

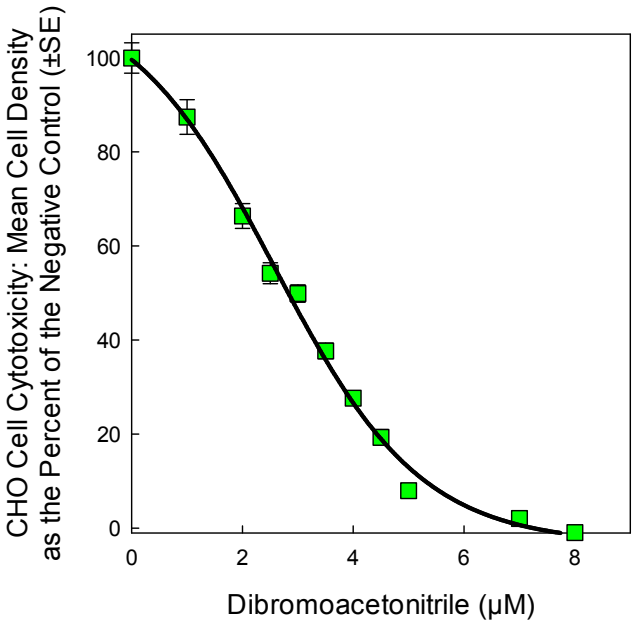

Figure S5. CHO cell cytotoxicity concentration-response curve for DBAN. Mean (±SE) LC_{50} value was 2.79 ± 0.09 µM.

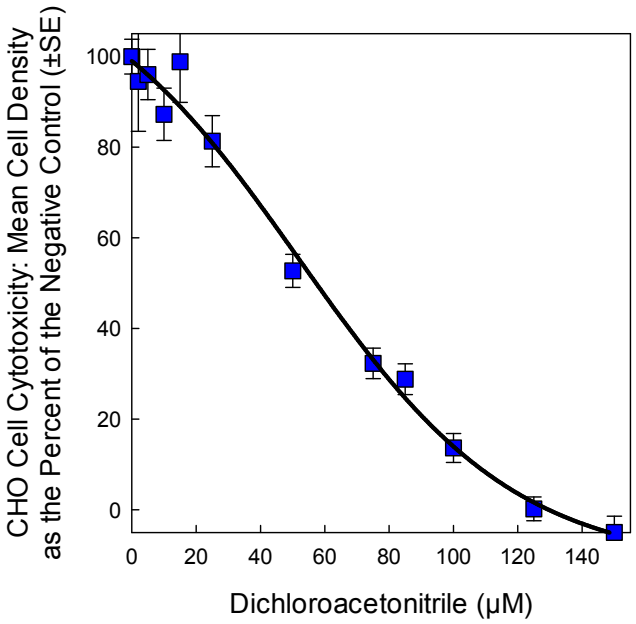

Figure S6. CHO cell cytotoxicity concentration-response curve for DCAN. Mean (±SE) LC_{50} value was 55.03 ± 3.23 µM.

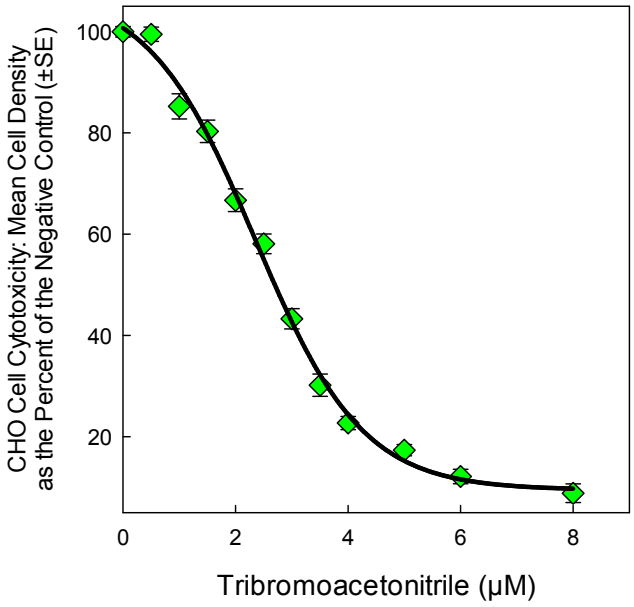

Figure S7. CHO cell cytotoxicity concentration-response curve for TBAN. Mean (±SE) LC_{50} value was 2.71 ± 0.04 µM.

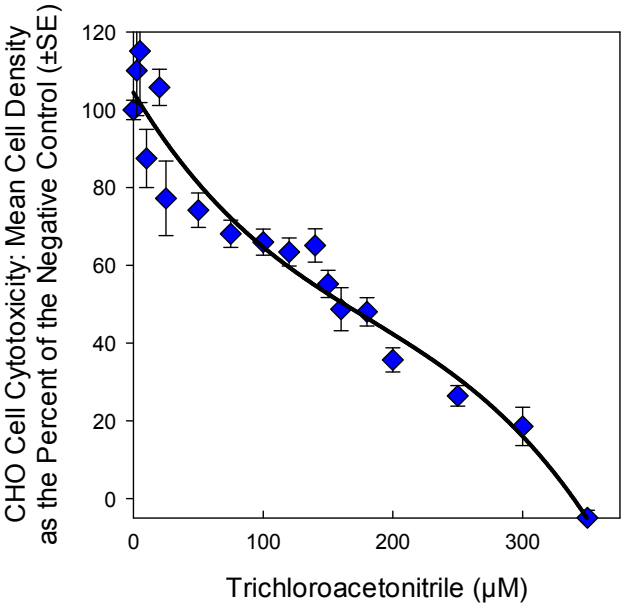

Figure S8. CHO cell cytotoxicity concentration-response curve for TCAN. Mean (±SE) LC_{50} value was 158.55 ± 6.01 µM.

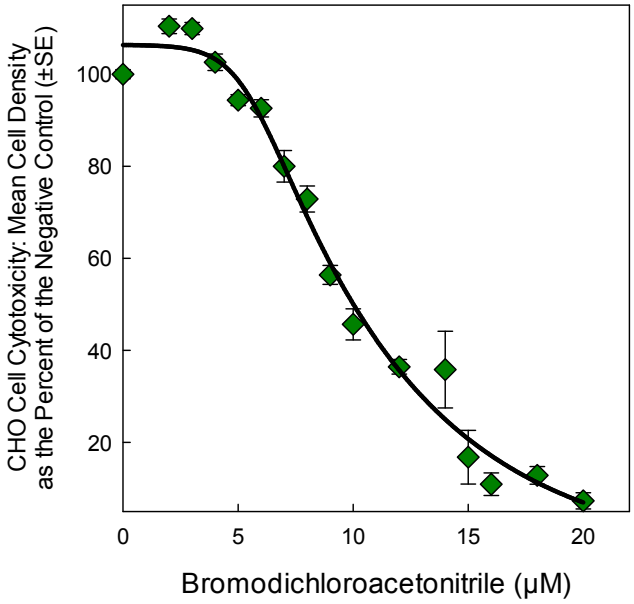

Figure S9. CHO cell cytotoxicity concentration-response curve for BDCAN. Mean (±SE) LC_{50} value was 10.22 ± 0.12 µM.

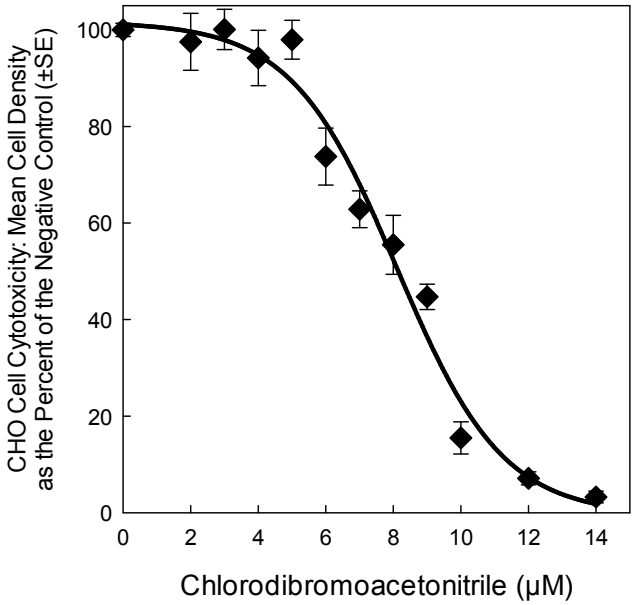

Figure S10. CHO cell cytotoxicity concentration-response curve for CDBAN. Mean (±SE) LC_{50} value was 8.14 ± 0.18 µM.

The ANOVA test statistic to determine significant decreases in cell viability for the three newly evaluated HANs are presented in Table S2 (TBAN), Table S3 (BDCAN) and Table S4 (CDBAN).

Table S2. One Way Analysis of Variance: Tribromoacetonitrile (TBAN) CHO cell cytotoxicity. Percent of the negative control.

Group Name	N	Missing	Mean	Std Dev	SEM
0 TBAN µM	24	0	99.974	5.116	1.044
0.5 TBAN µM	4	0	99.478	2.826	1.413
1 TBAN µM	8	0	85.243	7.056	2.495
1.5 TBAN µM	8	0	80.312	6.252	2.210
2 TBAN µM	10	0	66.713	7.067	2.235
2.5 TBAN µM	8	0	58.093	5.508	1.948
3 TBAN µM	8	0	43.266	5.667	2.003
3.5 TBAN µM	8	0	30.159	6.233	2.204
4 TBAN µM	10	0	22.686	4.133	1.307
5 TBAN µM	8	0	17.336	3.137	1.109
6 TBAN µM	10	0	12.131	4.548	1.438
8 TBAN µM	6	0	8.824	4.506	1.840

Source of Variation	DF	SS	MS	F	P
Between Groups	11	128912.588	11719.326	401.784	<0.001
Residual	100	2916.820	29.168		
Total	111	131829.409			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 TBAN µM vs. 6 TBAN µM	87.843	43.213	<0.001	Yes
0 TBAN µM vs. 4 TBAN µM	77.288	38.021	<0.001	Yes
0 TBAN µM vs. 5 TBAN µM	82.639	37.480	<0.001	Yes
0 TBAN µM vs. 8 TBAN µM	91.150	36.976	<0.001	Yes
0 TBAN µM vs. 3.5 TBAN µM	69.815	31.664	<0.001	Yes
0 TBAN µM vs. 3 TBAN µM	56.709	25.720	<0.001	Yes
0 TBAN µM vs. 2.5 TBAN µM	41.881	18.995	<0.001	Yes
0 TBAN µM vs. 2 TBAN µM	33.261	16.363	<0.001	Yes
0 TBAN µM vs. 1.5 TBAN µM	19.662	8.918	<0.001	Yes
0 TBAN µM vs. 1 TBAN µM	14.731	6.681	<0.001	Yes
0 TBAN µM vs. 0.5 TBAN µM	0.497	0.170	0.865	No

Table S3. One Way Analysis of Variance: Bromodichloroacetonitrile (BDCAN) CHO cell cytotoxicity. Percent of the negative control.

Group Name	N	Missing	Mean	Std Dev	SEM
0 BDCAN	32	0	100.007	5.657	1.000
2 BDCAN	9	0	110.412	4.775	1.592
3 BDCAN	4	0	109.911	2.635	1.318
4 BDCAN	13	0	102.589	6.452	1.790
5 BDCAN	8	0	94.370	3.412	1.206
6 BDCAN	17	0	92.592	7.649	1.855
7 BDCAN	4	0	79.994	6.856	3.428
8 BDCAN	13	0	72.903	10.203	2.830
9 BDCAN	4	0	56.405	4.114	2.057
10 BDCAN	9	0	45.637	10.176	3.392
12 BDCAN	8	0	36.424	4.518	1.597
14 BDCAN	8	0	35.808	23.578	8.336
15 BDCAN	5	0	16.787	13.050	5.836
16 BDCAN	4	0	10.924	4.901	2.451
18 BDCAN	4	0	12.848	3.847	1.924
20 BDCAN	5	0	7.322	3.932	1.758

Source of Variation	DF	SS	MS	F	P
Between Groups	15	159535.937	10635.729	141.080	<0.001
Residual	131	9875.827	75.388		
Total	146	169411.764			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 BDCAN vs. 20 BDCAN	92.685	22.198	<0.001	Yes
0 BDCAN vs. 15 BDCAN	83.220	19.931	<0.001	Yes
0 BDCAN vs. 16 BDCAN	89.083	19.346	<0.001	Yes
0 BDCAN vs. 18 BDCAN	87.159	18.928	<0.001	Yes
0 BDCAN vs. 14 BDCAN	64.200	18.706	<0.001	Yes
0 BDCAN vs. 12 BDCAN	63.583	18.526	<0.001	Yes
0 BDCAN vs. 10 BDCAN	54.370	16.596	<0.001	Yes
0 BDCAN vs. 8 BDCAN	27.104	9.491	<0.001	Yes
0 BDCAN vs. 9 BDCAN	43.602	9.469	<0.001	Yes
0 BDCAN vs. 7 BDCAN	20.014	4.346	<0.001	Yes
0 BDCAN vs. 2 BDCAN	10.405	3.176	0.009	Yes
0 BDCAN vs. 6 BDCAN	7.415	2.846	0.020	Yes
0 BDCAN vs. 3 BDCAN	9.904	2.151	0.097	No
0 BDCAN vs. 5 BDCAN	5.637	1.642	0.195	No
0 BDCAN vs. 4 BDCAN	2.582	0.904	0.368	No

Table S4. One Way Analysis of Variance: Chlorodibromoacetonitrile (CDBAN) CHO cell cytotoxicity. Percent of the negative control.

Group Name	N	Missing	Mean	Std Dev	SEM
0uM CDBAN	16	0	100.007	5.512	1.378
2 CDBAN	5	0	97.517	13.185	5.897
3 CDBAN	4	0	100.095	8.360	4.180
4 CDBAN	9	0	94.191	17.199	5.733
5 CDBAN	8	0	97.966	11.399	4.030
6 CDBAN	9	0	73.771	17.656	5.885
7 CDBAN	4	0	62.866	7.620	3.810
8 CDBAN	9	0	55.506	18.335	6.112
9 CDBAN	4	0	44.707	5.272	2.636
10 CDBAN	9	0	15.487	9.994	3.331
12 CDBAN	9	0	7.124	4.014	1.338
14 CDBAN	5	0	3.261	2.716	1.215

Source of Variation	DF	SS	MS	F	P
Between Groups	11	120619.108	10965.373	78.499	<0.001
Residual	79	11035.320	139.688		
Total	90	131654.428			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 CDBAN vs. 12 CDBAN	92.883	18.861	<0.001	Yes
0 CDBAN vs. 10 CDBAN	84.521	17.163	<0.001	Yes
0 CDBAN vs. 14 CDBAN	96.746	15.977	<0.001	Yes
0 CDBAN vs. 8 CDBAN	44.502	9.037	<0.001	Yes
0 CDBAN vs. 9 CDBAN	55.301	8.370	<0.001	Yes
0 CDBAN vs. 7 CDBAN	37.141	5.621	<0.001	Yes
0 CDBAN vs. 6 CDBAN	26.236	5.328	<0.001	Yes
0 CDBAN vs. 4 CDBAN	5.817	1.181	0.668	No
0 CDBAN vs. 2 CDBAN	2.490	0.411	0.968	No
0 CDBAN vs. 5 CDBAN	2.042	0.399	0.905	No
0 CDBAN vs. 3 CDBAN	0.0874	0.0132	0.989	No

Table S5. One Way Analysis of Variance: 10 HAN CTI Comparisons.

Group Name	N	Missing	Mean	Std Dev	SEM
IAN CTI	17	0	307.280	18.180	4.409
BAN CTI	25	0	325.828	35.268	7.054
DBAN CTI	16	0	364.565	47.938	11.985
BCAN CTI	19	0	130.839	35.932	8.243
TBAN CTI	14	0	369.561	20.086	5.368
CAN CTI	19	0	15.298	1.659	0.381
DCAN CTI	19	0	19.483	5.614	1.288
TCAN CTI	27	0	6.546	1.293	0.249
BDCAN CTI	18	0	98.097	4.737	1.117
CDBAN CTI	11	0	123.593	10.668	3.217

Source of Variation	DF	SS	MS	F	P
Between Groups	9	3840984.828	426776.092	744.800	<0.001
Residual	175	100276.348	573.008		
Total	184	3941261.176			

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

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
BAN CTI vs. TCAN CTI	319.282	48.056	<0.001	Yes
DBAN CTI vs. TCAN CTI	358.019	47.406	<0.001	Yes
TBAN CTI vs. TCAN CTI	363.014	46.047	<0.001	Yes
DBAN CTI vs. CAN CTI	349.267	43.001	<0.001	Yes
BAN CTI vs. CAN CTI	310.530	42.623	<0.001	Yes
DBAN CTI vs. DCAN CTI	345.082	42.486	<0.001	Yes
BAN CTI vs. DCAN CTI	306.345	42.048	<0.001	Yes
TBAN CTI vs. CAN CTI	354.262	42.017	<0.001	Yes
TBAN CTI vs. DCAN CTI	350.077	41.521	<0.001	Yes
IAN CTI vs. TCAN CTI	300.734	40.577	<0.001	Yes
IAN CTI vs. CAN CTI	291.982	36.536	<0.001	Yes
IAN CTI vs. DCAN CTI	287.797	36.013	<0.001	Yes
DBAN CTI vs. BDCAN CTI	266.469	32.398	<0.001	Yes
TBAN CTI vs. BDCAN CTI	271.464	31.824	<0.001	Yes
BAN CTI vs. BDCAN CTI	227.731	30.776	<0.001	Yes
DBAN CTI vs. BCAN CTI	233.727	28.776	<0.001	Yes
TBAN CTI vs. BCAN CTI	238.722	28.314	<0.001	Yes
BAN CTI vs. BCAN CTI	194.989	26.764	<0.001	Yes
IAN CTI vs. BDCAN CTI	209.184	25.839	<0.001	Yes
DBAN CTI vs. CDBAN CTI	240.973	25.702	<0.001	Yes
TBAN CTI vs. CDBAN CTI	245.968	25.503	<0.001	Yes
BAN CTI vs. CDBAN CTI	202.235	23.350	<0.001	Yes
IAN CTI vs. BCAN CTI	176.441	22.079	<0.001	Yes
IAN CTI vs. CDBAN CTI	183.687	19.831	<0.001	Yes
BCAN CTI vs. TCAN CTI	124.293	17.340	<0.001	Yes
BCAN CTI vs. CAN CTI	115.541	14.877	<0.001	Yes

BCAN CTI vs. DCAN CTI	111.355	14.338	<0.001	Yes
CDBAN CTI vs. TCAN CTI	117.047	13.670	<0.001	Yes
BDCAN CTI vs. TCAN CTI	91.550	12.569	<0.001	Yes
CDBAN CTI vs. CAN CTI	108.295	11.941	<0.001	Yes
CDBAN CTI vs. DCAN CTI	104.109	11.479	<0.001	Yes
BDCAN CTI vs. CAN CTI	82.798	10.516	<0.001	Yes
BDCAN CTI vs. DCAN CTI	78.613	9.985	<0.001	Yes
TBAN CTI vs. IAN CTI	62.280	7.209	<0.001	Yes
DBAN CTI vs. IAN CTI	57.285	6.871	<0.001	Yes
TBAN CTI vs. BAN CTI	43.733	5.473	<0.001	Yes
DBAN CTI vs. BAN CTI	38.738	5.055	<0.001	Yes
BCAN CTI vs. BDCAN CTI	32.742	4.159	<0.001	Yes
CDBAN CTI vs. BDCAN CTI	25.496	2.783	0.041	Yes
BAN CTI vs. IAN CTI	18.548	2.465	0.085	No
DCAN CTI vs. TCAN CTI	12.937	1.805	0.315	No
CAN CTI vs. TCAN CTI	8.752	1.221	0.637	No
BCAN CTI vs. CDBAN CTI	7.246	0.799	0.810	No
TBAN CTI vs. DBAN CTI	4.995	0.570	0.814	No
DCAN CTI vs. CAN CTI	4.185	0.539	0.591	No

Single cell gel electrophoresis genotoxicity analyses. Single cell gel electrophoresis (SCGE or comet) quantitatively measures genomic DNA damage including DNA strand breaks, alkali-labile sites, incomplete excision repair sites, and interstrand crosslinks in the nuclei of cells.[14-16] CHO cells were treated for 4 h with a minimum of 10 concentrations; a range finding experiment plus a minimum of two repeated experiments were conducted. The biological metric was the average %Tail DNA value; a regression analysis of the SCGE concentration-response curve was conducted to obtain the concentration that induced a 50%Tail DNA value. The details of SCGE analyses were published.[11]

The concentration-response graphs illustrating the CHO cell SCGE genomic DNA damage of the HANs are presented in Figures S11 to S20.

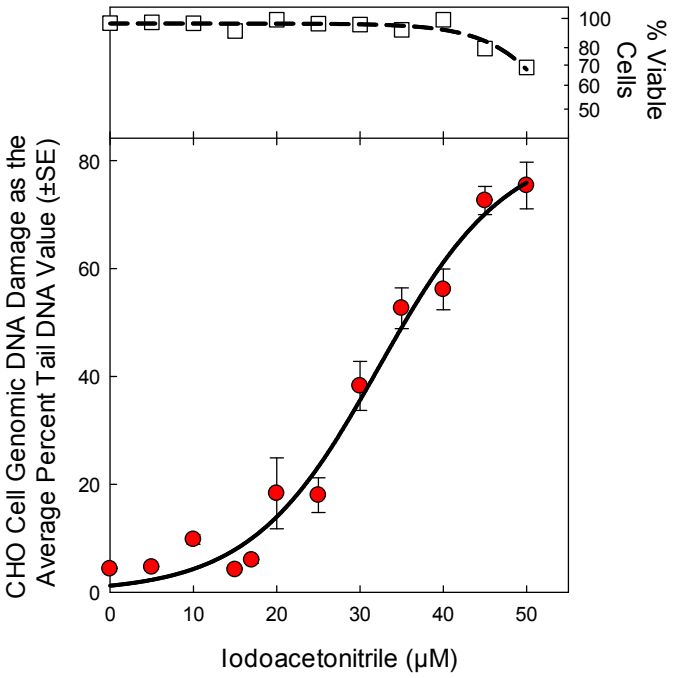

Figure S11. CHO cell genotoxicity concentration-response curve for IAN. The top panel illustrates the acute cytotoxicity and the bottom panel presents the genotoxicity as the Mean (±SE) 50% Tail DNA value that was 34.24 ± 0.76 μM.

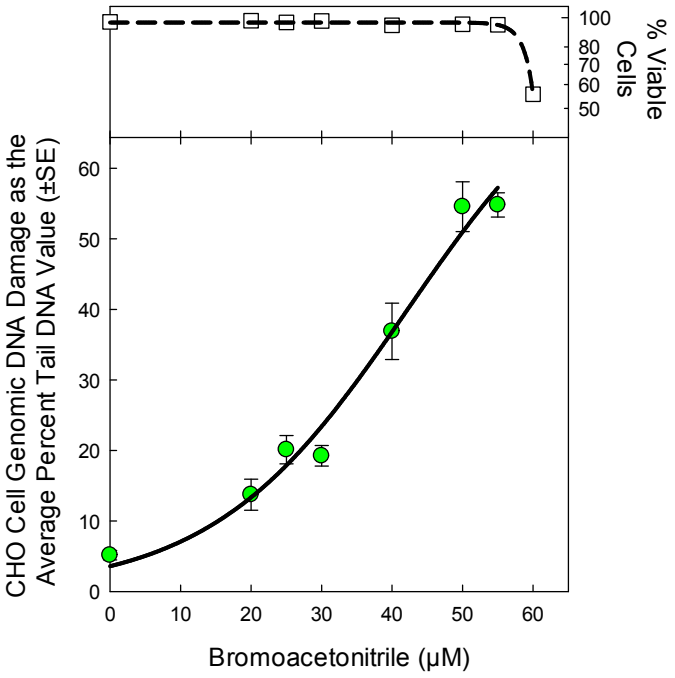

Figure S12. CHO cell genotoxicity concentration-response curve for BAN. The top panel illustrates the acute cytotoxicity and the bottom panel presents the genotoxicity as the Mean (±SE) 50% Tail DNA value that was 48.19 ± 1.06 μM.

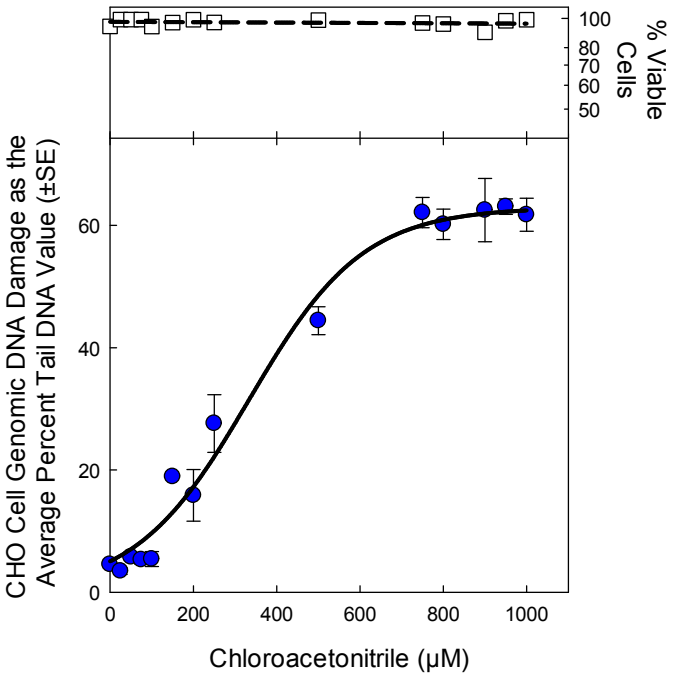

Figure S13. CHO cell genotoxicity concentration-response curve for CAN. The top panel illustrates the acute cytotoxicity and the bottom panel presents the genotoxicity as the Mean (±SE) 50% Tail DNA value that was 517.9 ± 26.2 µM.

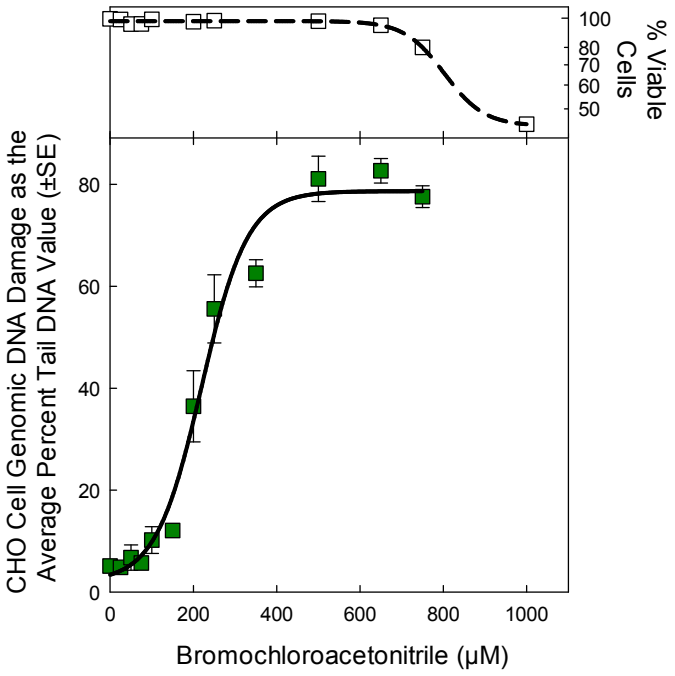

Figure S14. CHO cell genotoxicity concentration-response curve for BCAN. The top panel illustrates the acute cytotoxicity and the bottom panel presents the genotoxicity as the Mean (±SE) 50% Tail DNA value that was 250.1 ± 12.3 µM.

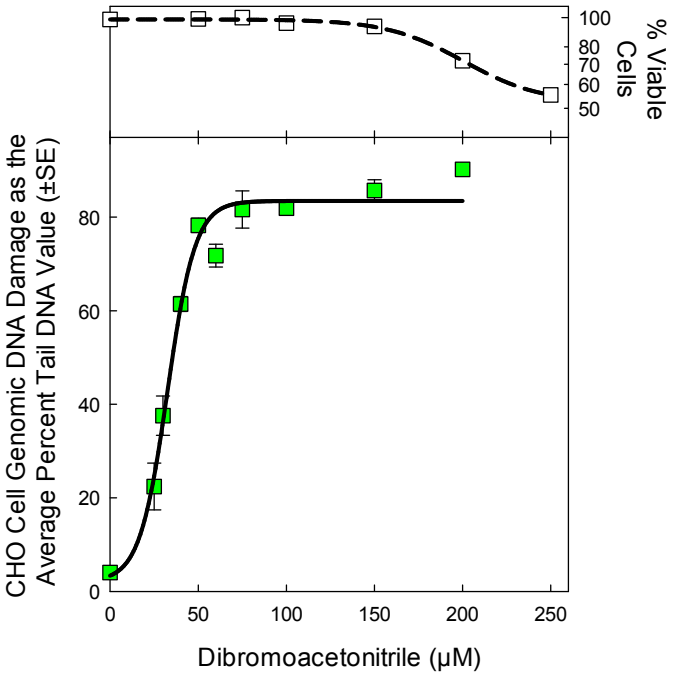

Figure S15. CHO cell genotoxicity concentration-response curve for DBAN. The top panel illustrates the acute cytotoxicity and the bottom panel presents the genotoxicity as the Mean (±SE) 50% Tail DNA value that was 35.50 ± 0.37 µM.

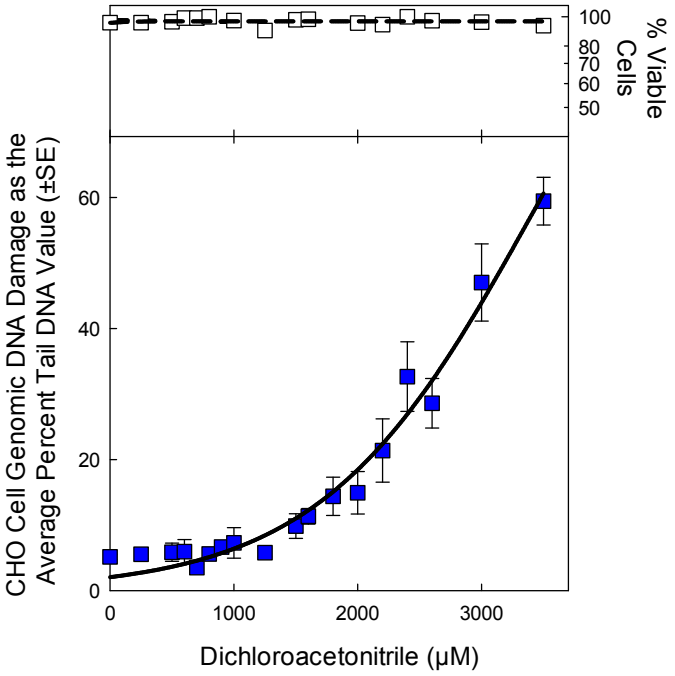

Figure S16. CHO cell genotoxicity concentration-response curve for DCAN. The top panel illustrates the acute cytotoxicity and the bottom panel presents the genotoxicity as the Mean (±SE) 50% Tail DNA value that was 3083 ± 85.4 µM.

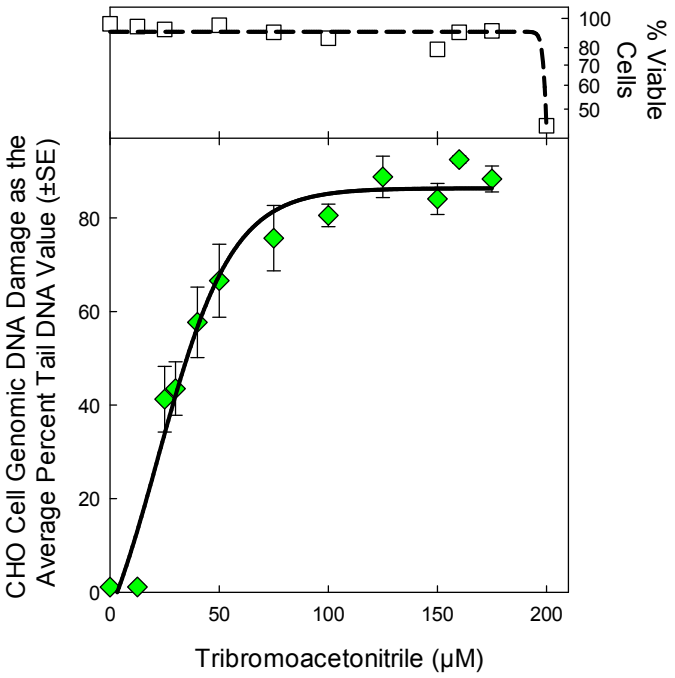

Figure S17. CHO cell genotoxicity concentration-response curve for TBAN. The top panel illustrates the acute cytotoxicity and the bottom panel presents the genotoxicity as the Mean (±SE) 50% Tail DNA value that was 37.76 ± 3.23 µM.

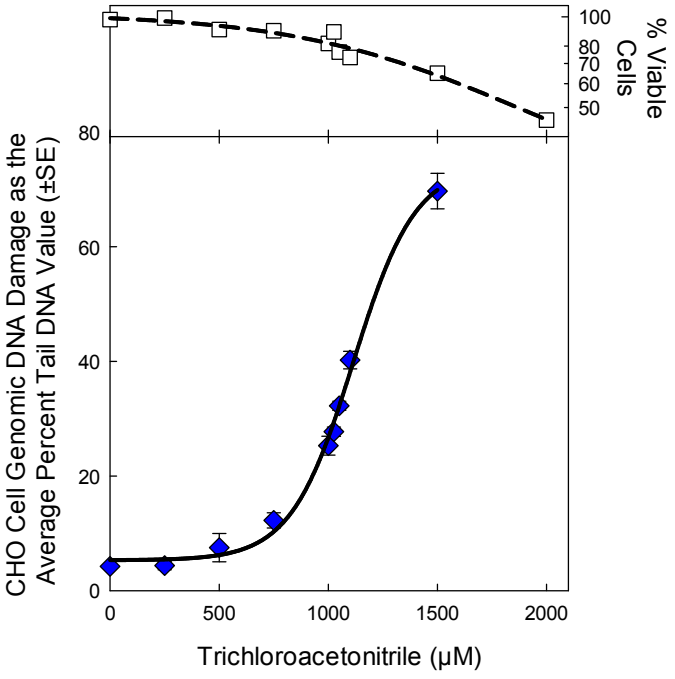

Figure S18. CHO cell genotoxicity concentration-response curve for TCAN. The top panel illustrates the acute cytotoxicity and the bottom panel presents the genotoxicity as the Mean (±SE) 50% Tail DNA value that was 1187 ± 15.2 µM.

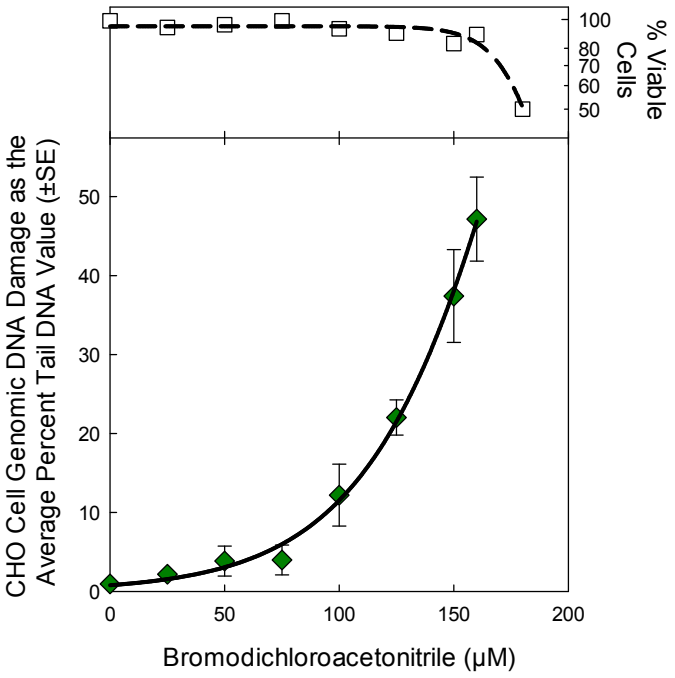

Figure S19. CHO cell genotoxicity concentration-response curve for BDCAN. The top panel illustrates the acute cytotoxicity and the bottom panel presents the genotoxicity as the Mean (±SE) 50% Tail DNA value that was 163.29 ± 1.45 μM.

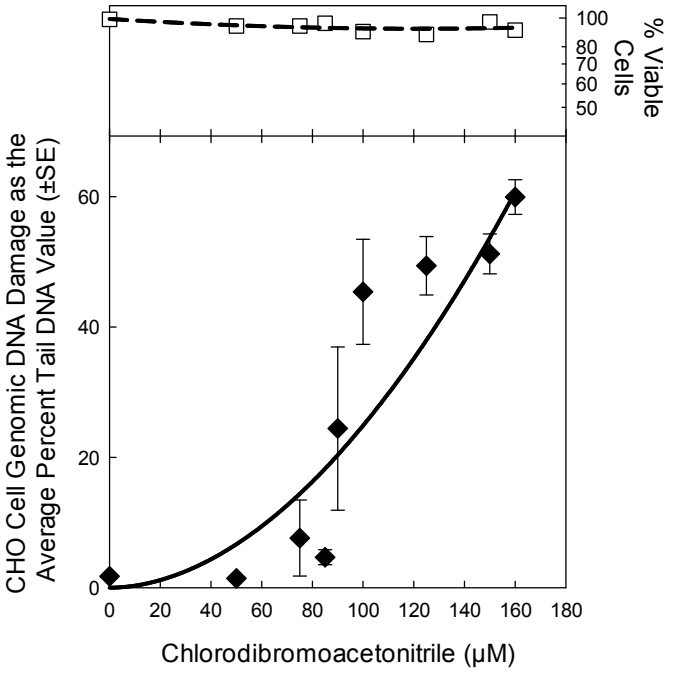

Figure S20. CHO cell genotoxicity concentration-response curve for CDBAN. The top panel illustrates the acute cytotoxicity and the bottom panel presents the genotoxicity as the Mean (±SE) 50% Tail DNA value that was 139.73 ± 2.89 μM.

The ANOVA test statistic to determine if a significant increase in genomic DNA damage (%TDNA) over their concurrent negative control for the HANs are presented in Tables S6 – Table S15.

Table S6. One Way Analysis of Variance: Iodoacetonitrile (IAN) CHO cell genotoxicity SCGE %Tail DNA.

Group Name	N	Missing	Mean	Std Dev	SEM
IAN 0 µM	8	0	4.325	1.105	0.391
IAN 5	2	0	4.656	0.507	0.358
IAN 10	2	0	9.779	1.234	0.872
IAN 15	4	0	4.194	0.862	0.431
IAN 17	6	0	5.968	1.444	0.590
IAN 20	6	0	18.331	16.081	6.565
IAN 25	6	0	17.993	7.881	3.217
IAN 30	6	0	38.251	11.131	4.544
IAN 35	8	0	52.653	10.681	3.776
IAN 40	6	0	56.148	9.226	3.766
IAN 45	10	0	72.612	8.229	2.602
IAN 50	4	0	75.403	8.648	4.324

Source of Variation	DF	SS	MS	F	P
Between Groups	11	48799.515	4436.320	57.723	<0.001
Residual	56	4303.882	76.855		
Total	67	53103.396			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
IAN 0 µM vs. IAN 45	68.287	16.421	<0.001	Yes
IAN 0 µM vs. IAN 50	71.079	13.240	<0.001	Yes
IAN 0 µM vs. IAN 35	48.328	11.025	<0.001	Yes
IAN 0 µM vs. IAN 40	51.824	10.946	<0.001	Yes
IAN 0 µM vs. IAN 30	33.926	7.166	<0.001	Yes
IAN 0 µM vs. IAN 20	14.006	2.958	0.027	Yes
IAN 0 µM vs. IAN 25	13.668	2.887	0.027	Yes
IAN 0 µM vs. IAN 10	5.454	0.787	0.898	No
IAN 0 µM vs. IAN 17	1.643	0.347	0.980	No
IAN 0 µM vs. IAN 5	0.332	0.0479	0.999	No
IAN 0 µM vs. IAN 15	0.130	0.0243	0.981	No

Table S7. One Way Analysis of Variance: Bromoacetonitrile (BAN) CHO cell genotoxicity SCGE %Tail DNA.

Group Name	N	Missing	Mean	Std Dev	SEM
BAN 0 µM	8	0	5.151	1.878	0.664
BAN 20	4	0	13.727	4.414	2.207
BAN 25	6	0	20.110	4.909	2.004
BAN 30	6	0	19.237	3.575	1.459
BAN 40	6	0	36.887	9.771	3.989
BAN 50	7	0	54.552	9.332	3.527
BAN 55	6	0	54.809	4.210	1.719

Source of Variation	DF	SS	MS	F	P
Between Groups	6	15462.675	2577.113	68.419	<0.001
Residual	36	1356.006	37.667		
Total	42	16818.681			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
BAN 0 µM vs. BAN 50	49.401	15.553	<0.001	Yes
BAN 0 µM vs. BAN 55	49.658	14.982	<0.001	Yes
BAN 0 µM vs. BAN 40	31.736	9.575	<0.001	Yes
BAN 0 µM vs. BAN 25	14.959	4.513	<0.001	Yes
BAN 0 µM vs. BAN 30	14.086	4.250	<0.001	Yes
BAN 0 µM vs. BAN 20	8.576	2.282	0.029	Yes

Table S8. One Way Analysis of Variance: Chloroacetonitrile (CAN) CHO cell genotoxicity SCGE %Tail DNA.

Group Name	N	Missing	Mean	Std Dev	SEM
CAN 0 µM	5	0	4.561	0.700	0.313
CAN 25	2	0	3.464	0.770	0.544
CAN 50	2	0	5.774	0.000	0.000
CAN 75	2	0	5.330	1.051	0.743
CAN 100	3	0	5.429	2.112	1.219
CAN 150	2	0	18.919	0.000	0.000
CAN 200	4	0	15.841	8.438	4.219
CAN 250	6	0	27.600	11.586	4.730
CAN 500	6	0	44.428	5.585	2.280
CAN 750	6	0	62.103	6.059	2.474
CAN 800	6	0	60.184	6.105	2.492
CAN 900	6	0	62.503	12.683	5.178
CAN 950	2	0	63.072	1.779	1.258
CAN 1000	6	0	61.750	6.611	2.699

Source of Variation	DF	SS	MS	F	P
Between Groups	13	33642.338	2587.872	46.490	<0.001
Residual	44	2449.285	55.666		
Total	57	36091.623			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
CAN 0 µM vs. CAN 900	57.943	12.825	<0.001	Yes
CAN 0 µM vs. CAN 750	57.542	12.737	<0.001	Yes
CAN 0 µM vs. CAN 1000	57.190	12.659	<0.001	Yes
CAN 0 µM vs. CAN 800	55.623	12.312	<0.001	Yes
CAN 0 µM vs. CAN 950	58.511	9.373	<0.001	Yes
CAN 0 µM vs. CAN 500	39.867	8.824	<0.001	Yes
CAN 0 µM vs. CAN 250	23.039	5.100	<0.001	Yes
CAN 0 µM vs. CAN 150	14.359	2.300	0.147	No
CAN 0 µM vs. CAN 200	11.280	2.254	0.138	No
CAN 0 µM vs. CAN 50	1.213	0.194	0.999	No
CAN 0 µM vs. CAN 25	1.097	0.176	0.997	No
CAN 0 µM vs. CAN 100	0.868	0.159	0.984	No
CAN 0 µM vs. CAN 75	0.769	0.123	0.903	No

Table S9. One Way Analysis of Variance: Bromochloroacetonitrile (BCAN) CHO cell genotoxicity SCGE %Tail DNA.

Group Name	N	Missing	Mean	Std Dev	SEM
BCAN 0 µM	6	0	5.093	1.420	0.580
BCAN 25	2	0	4.835	0.897	0.635
BCAN 50	2	0	6.761	3.501	2.476
BCAN 75	2	0	5.700	0.731	0.517
BCAN 100	4	0	10.190	5.251	2.625
BCAN 150	6	0	12.061	3.209	1.310
BCAN 200	6	0	36.449	17.118	6.988
BCAN 250	6	0	55.574	16.365	6.681
BCAN 350	6	0	62.565	6.521	2.662
BCAN 500	6	0	81.086	10.892	4.447
BCAN 650	6	0	82.674	5.914	2.415
BCAN 750	4	0	77.587	4.281	2.141

Source of Variation	DF	SS	MS	F	P
Between Groups	11	53426.540	4856.958	53.455	<0.001
Residual	44	3997.866	90.861		
Total	55	57424.406			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
BCAN 0 µM vs. BCAN 650	77.581	14.097	<0.001	Yes
BCAN 0 µM vs. BCAN 500	75.992	13.808	<0.001	Yes
BCAN 0 µM vs. BCAN 750	72.493	11.782	<0.001	Yes
BCAN 0 µM vs. BCAN 350	57.471	10.443	<0.001	Yes
BCAN 0 µM vs. BCAN 250	50.481	9.173	<0.001	Yes
BCAN 0 µM vs. BCAN 200	31.356	5.698	<0.001	Yes
BCAN 0 µM vs. BCAN 150	6.967	1.266	0.696	No
BCAN 0 µM vs. BCAN 100	5.096	0.828	0.880	No
BCAN 0 µM vs. BCAN 50	1.668	0.214	0.995	No
BCAN 0 µM vs. BCAN 75	0.606	0.0779	0.996	No
BCAN 0 µM vs. BCAN 25	0.258	0.0332	0.974	No

Table S10. One Way Analysis of Variance: Dibromoacetonitrile (DBAN) CHO cell genotoxicity SCGE %Tail DNA.

Group Name	N	Missing	Mean	Std Dev	SEM
DBAN 0 µM	6	0	4.056	1.114	0.455
DBAN 25	2	0	22.427	7.081	5.007
DBAN 30	2	0	37.559	5.935	4.197
DBAN 40	2	0	61.450	2.251	1.592
DBAN 50	8	0	78.244	4.248	1.502
DBAN 60	2	0	69.927	1.935	1.368
DBAN 75	6	0	81.632	9.744	3.978
DBAN 100	12	0	82.427	2.748	0.793
DBAN 150	6	0	85.688	5.653	2.308
DBAN 200	4	0	90.201	1.262	0.631

Source of Variation	DF	SS	MS	F	P
Between Groups	9	38930.391	4325.599	182.313	<0.001
Residual	40	949.047	23.726		
Total	49	39879.438			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
DBAN 0 µM vs. DBAN 100	78.371	32.179	<0.001	Yes
DBAN 0 µM vs. DBAN 150	81.632	29.027	<0.001	Yes
DBAN 0 µM vs. DBAN 50	74.187	28.202	<0.001	Yes
DBAN 0 µM vs. DBAN 75	77.576	27.585	<0.001	Yes
DBAN 0 µM vs. DBAN 200	86.145	27.398	<0.001	Yes
DBAN 0 µM vs. DBAN 60	65.871	16.562	<0.001	Yes
DBAN 0 µM vs. DBAN 40	57.394	14.431	<0.001	Yes
DBAN 0 µM vs. DBAN 30	33.503	8.424	<0.001	Yes
DBAN 0 µM vs. DBAN 25	18.371	4.619	<0.001	Yes

Table S11. One Way Analysis of Variance: Dichloroacetonitrile (DCAN) CHO cell genotoxicity SCGE %Tail DNA.

Group Name	N	Missing	Mean	Std Dev	SEM
DCAN 0 µM	8	0	5.150	1.150	0.407
DCAN 250	2	0	5.550	0.955	0.675
DCAN 500	2	0	5.859	1.964	1.389
DCAN 600	2	0	5.958	2.601	1.839
DCAN 700	2	0	3.503	0.713	0.504
DCAN 800	2	0	5.582	0.906	0.640
DCAN 900	2	0	6.651	0.308	0.218
DCAN 1000	2	0	7.287	3.298	2.332
DCAN 1250	4	0	5.795	1.286	0.643
DCAN 1500	6	0	9.848	4.613	1.883
DCAN 1600	6	0	11.341	2.903	1.185
DCAN 1800	6	0	14.381	7.152	2.920
DCAN 2000	6	0	14.925	7.947	3.244
DCAN 2200	6	0	21.373	11.836	4.832
DCAN 2400	6	0	32.670	13.014	5.313
DCAN 2600	6	0	28.597	9.284	3.790
DCAN 3000	6	0	47.039	14.420	5.887
DCAN 3500	6	0	59.444	8.916	3.640

Source of Variation	DF	SS	MS	F	P
Between Groups	17	21970.765	1292.398	19.199	<0.001
Residual	62	4173.536	67.315		
Total	79	26144.301			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
DCAN 0 µM vs. DCAN 3500	54.294	12.253	<0.001	Yes
DCAN 0 µM vs. DCAN 3000	41.889	9.454	<0.001	Yes
DCAN 0 µM vs. DCAN 2400	27.520	6.211	<0.001	Yes
DCAN 0 µM vs. DCAN 2600	23.447	5.292	<0.001	Yes
DCAN 0 µM vs. DCAN 2200	16.223	3.661	0.007	Yes
DCAN 0 µM vs. DCAN 2000	9.774	2.206	0.316	No
DCAN 0 µM vs. DCAN 1800	9.231	2.083	0.372	No
DCAN 0 µM vs. DCAN 1600	6.191	1.397	0.840	No
DCAN 0 µM vs. DCAN 1500	4.698	1.060	0.956	No
DCAN 0 µM vs. DCAN 1000	2.137	0.329	1.000	No
DCAN 0 µM vs. DCAN 700	1.647	0.254	1.000	No
DCAN 0 µM vs. DCAN 900	1.501	0.231	1.000	No
DCAN 0 µM vs. DCAN 1250	0.645	0.128	1.000	No
DCAN 0 µM vs. DCAN 600	0.808	0.125	1.000	No
DCAN 0 µM vs. DCAN 500	0.709	0.109	0.999	No
DCAN 0 µM vs. DCAN 800	0.432	0.0666	0.997	No
DCAN 0 µM vs. DCAN 250	0.400	0.0617	0.951	No

Table S12. One Way Analysis of Variance: Tribromoacetonitrile (TBAN) CHO cell genotoxicity SCGE %Tail DNA.

Group Name	N	Missing	Mean	Std Dev	SEM
TBAN 0 µM	8	0	1.097	0.866	0.306
TBAN 12.5	2	0	1.134	0.785	0.555
TBAN 25	9	0	41.263	21.067	7.022
TBAN 30	9	0	43.533	17.197	5.732
TBAN 40	7	0	57.707	19.929	7.532
TBAN 50	7	0	66.584	20.603	7.787
TBAN 75	5	0	75.683	15.594	6.974
TBAN 100	4	0	80.542	4.796	2.398
TBAN 125	2	0	88.778	6.257	4.425
TBAN 150	4	0	84.061	6.629	3.315
TBAN 160	4	0	92.461	1.588	0.794
TBAN 175	2	0	88.321	3.930	2.779

Source of Variation	DF	SS	MS	F	P
Between Groups	11	51105.081	4645.916	19.602	<0.001
Residual	51	12087.810	237.016		
Total	62	63192.891			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
TBAN 0 µM vs. TBAN 160	91.364	9.691	<0.001	Yes
TBAN 0 µM vs. TBAN 150	82.964	8.800	<0.001	Yes
TBAN 0 µM vs. TBAN 75	74.586	8.498	<0.001	Yes
TBAN 0 µM vs. TBAN 100	79.445	8.427	<0.001	Yes
TBAN 0 µM vs. TBAN 50	65.487	8.219	<0.001	Yes
TBAN 0 µM vs. TBAN 125	87.681	7.204	<0.001	Yes
TBAN 0 µM vs. TBAN 175	87.224	7.166	<0.001	Yes
TBAN 0 µM vs. TBAN 40	56.609	7.105	<0.001	Yes
TBAN 0 µM vs. TBAN 30	42.436	5.673	<0.001	Yes
TBAN 0 µM vs. TBAN 25	40.166	5.369	<0.001	Yes
TBAN 0 µM vs. TBAN 12.5	0.0370	0.00304	0.998	No

Table S13. One Way Analysis of Variance: Trichloroacetonitrile (TCAN) CHO cell genotoxicity SCGE %Tail DNA.

Group Name	N	Missing	Mean	Std Dev	SEM
TCAN 0 µM	6	0	4.247	0.454	0.185
TCAN 250	2	0	4.379	1.034	0.731
TCAN 500	2	0	7.509	3.486	2.465
TCAN 750	6	0	12.253	3.246	1.325
TCAN 1000	6	0	25.298	4.041	1.650
TCAN 1025	6	0	27.733	1.993	0.814
TCAN 1050	6	0	32.247	1.920	0.784
TCAN 1100	6	0	40.234	3.747	1.530
TCAN 1500	2	0	69.713	4.380	3.097

Source of Variation	DF	SS	MS	F	P
Between Groups	8	10744.004	1343.001	160.401	<0.001
Residual	33	276.302	8.373		
Total	41	11020.306			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
TCAN 0 µM vs. TCAN 1500	65.466	27.709	<0.001	Yes
TCAN 0 µM vs. TCAN 1100	35.987	21.541	<0.001	Yes
TCAN 0 µM vs. TCAN 1050	28.000	16.760	<0.001	Yes
TCAN 0 µM vs. TCAN 1025	23.486	14.058	<0.001	Yes
TCAN 0 µM vs. TCAN 1000	21.051	12.601	<0.001	Yes
TCAN 0 µM vs. TCAN 750	8.006	4.792	<0.001	Yes
TCAN 0 µM vs. TCAN 500	3.261	1.380	0.322	No
TCAN 0 µM vs. TCAN 250	0.132	0.0557	0.956	No

Table S14. One Way Analysis of Variance: Bromodichloroacetonitrile (BDCAN) CHO cell genotoxicity SCGE %Tail DNA.

Group Name	N	Missing	Mean	Std Dev	SEM
0 BDCAN µM	6	0	0.945	0.513	0.209
25 BDCAN	2	0	2.175	0.0288	0.0204
50 BDCAN	2	0	3.850	2.681	1.895
75 BDCAN	2	0	3.979	2.673	1.890
100 BDCAN	6	0	12.192	9.598	3.919
125 BDCAN	6	0	22.021	5.456	2.227
150 BDCAN	6	0	37.414	14.384	5.872
160 BDCAN	7	0	47.150	14.066	5.316

Source of Variation	DF	SS	MS	F	P
Between Groups	7	11162.753	1594.679	16.245	<0.001
Residual	29	2846.767	98.164		
Total	36	14009.520			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 BDCAN µM vs. 160 BDCAN	46.205	8.382	<0.001	Yes
0 BDCAN µM vs. 150 BDCAN	36.469	6.375	<0.001	Yes
0 BDCAN µM vs. 125 BDCAN	21.076	3.684	0.005	Yes
0 BDCAN µM vs. 100 BDCAN	11.247	1.966	0.216	No
0 BDCAN µM vs. 75 BDCAN	3.035	0.375	0.976	No
0 BDCAN µM vs. 50 BDCAN	2.905	0.359	0.923	No
0 BDCAN µM vs. 25 BDCAN	1.230	0.152	0.880	No

Table S15. One Way Analysis of Variance: Chlorodibromoacetonitrile (CDBAN) CHO cell genotoxicity SCGE %Tail DNA.

Group Name	N	Missing	Mean	Std Dev	SEM
0 μM CDBAN	7	0	1.756	0.674	0.255
50 CDBAN	3	0	1.427	0.496	0.286
75 CDBAN	3	0	7.617	10.102	5.832
85 CDBAN	4	0	4.677	2.303	1.152
90 CDBAN	2	0	24.425	17.715	12.526
100 CDBAN	6	0	45.406	19.735	8.057
125 CDBAN	9	0	49.414	13.436	4.479
150 CDBAN	10	0	51.228	9.701	3.068
160 CDBAN	6	0	59.954	6.529	2.665

Source of Variation	DF	SS	MS	F	P
Between Groups	8	26100.281	3262.535	26.812	<0.001
Residual	41	4988.874	121.680		
Total	49	31089.156			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 μM CDBAN vs. 160 CDBAN	58.198	9.483	<0.001	Yes
0 μM CDBAN vs. 150 CDBAN	49.472	9.101	<0.001	Yes
0 μM CDBAN vs. 125 CDBAN	47.658	8.573	<0.001	Yes
0 μM CDBAN vs. 100 CDBAN	43.650	7.113	<0.001	Yes
0 μM CDBAN vs. 90 CDBAN	22.669	2.563	0.055	No
0 μM CDBAN vs. 75 CDBAN	5.861	0.770	0.830	No
0 μM CDBAN vs. 85 CDBAN	2.921	0.422	0.894	No
0 μM CDBAN vs. 50 CDBAN	0.329	0.0432	0.966	No

Table S16. One Way Analysis of Variance: 10 HAN GTI Comparisons.

Group Name	N	Missing	Mean	Std Dev	SEM
IAN GTI	11	0	29.347	2.099	0.633
BAN GTI	11	0	20.854	1.572	0.474
DBAN GTI	15	0	28.210	1.141	0.295
BCAN GTI	11	0	4.110	0.752	0.227
TBAN GTI	11	0	28.372	7.550	2.276
CAN GTI	11	0	1.997	0.444	0.134
DCAN GTI	11	0	0.327	0.0292	0.00881
TCAN GTI	11	0	0.844	0.0342	0.0103
BDCAN GTI	11	0	6.129	0.180	0.0544
CDBAN GTI	11	0	7.187	0.484	0.146

Source of Variation	DF	SS	MS	F	P
Between Groups	9	16162.572	1795.841	279.887	<0.001
Residual	104	667.295	6.416		
Total	113	16829.867			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).. Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
DBAN GTI vs. DCAN GTI	27.883	27.730	<0.001	Yes
DBAN GTI vs. TCAN GTI	27.366	27.216	<0.001	Yes
IAN GTI vs. DCAN GTI	29.020	26.868	<0.001	Yes
IAN GTI vs. TCAN GTI	28.503	26.390	<0.001	Yes
DBAN GTI vs. CAN GTI	26.213	26.069	<0.001	Yes
TBAN GTI vs. DCAN GTI	28.045	25.965	<0.001	Yes
TBAN GTI vs. TCAN GTI	27.528	25.487	<0.001	Yes
IAN GTI vs. CAN GTI	27.350	25.322	<0.001	Yes
TBAN GTI vs. CAN GTI	26.375	24.419	<0.001	Yes
DBAN GTI vs. BCAN GTI	24.100	23.967	<0.001	Yes
IAN GTI vs. BCAN GTI	25.237	23.365	<0.001	Yes
TBAN GTI vs. BCAN GTI	24.262	22.463	<0.001	Yes
DBAN GTI vs. BDCAN GTI	22.081	21.960	<0.001	Yes
IAN GTI vs. BDCAN GTI	23.218	21.496	<0.001	Yes
DBAN GTI vs. CDBAN GTI	21.023	20.908	<0.001	Yes
TBAN GTI vs. BDCAN GTI	22.243	20.593	<0.001	Yes
IAN GTI vs. CDBAN GTI	22.160	20.517	<0.001	Yes
TBAN GTI vs. CDBAN GTI	21.185	19.614	<0.001	Yes
BAN GTI vs. DCAN GTI	20.527	19.005	<0.001	Yes
BAN GTI vs. TCAN GTI	20.010	18.526	<0.001	Yes
BAN GTI vs. CAN GTI	18.857	17.458	<0.001	Yes
BAN GTI vs. BCAN GTI	16.744	15.502	<0.001	Yes
BAN GTI vs. BDCAN GTI	14.725	13.633	<0.001	Yes
BAN GTI vs. CDBAN GTI	13.667	12.654	<0.001	Yes
IAN GTI vs. BAN GTI	8.493	7.863	<0.001	Yes
DBAN GTI vs. BAN GTI	7.356	7.316	<0.001	Yes
TBAN GTI vs. BAN GTI	7.518	6.961	<0.001	Yes
CDBAN GTI vs. DCAN GTI	6.860	6.351	<0.001	Yes

CDBAN GTI vs. TCAN GTI	6.343	5.873	<0.001	Yes
BDCAN GTI vs. DCAN GTI	5.802	5.372	<0.001	Yes
BDCAN GTI vs. TCAN GTI	5.285	4.893	<0.001	Yes
CDBAN GTI vs. CAN GTI	5.190	4.805	<0.001	Yes
BDCAN GTI vs. CAN GTI	4.132	3.826	0.003	Yes
BCAN GTI vs. DCAN GTI	3.783	3.503	0.008	Yes
BCAN GTI vs. TCAN GTI	3.266	3.024	0.034	Yes
CDBAN GTI vs. BCAN GTI	3.076	2.848	0.050	Yes
BCAN GTI vs. CAN GTI	2.113	1.957	0.388	No
BDCAN GTI vs. BCAN GTI	2.019	1.869	0.413	No
CAN GTI vs. DCAN GTI	1.670	1.546	0.608	No
IAN GTI vs. DBAN GTI	1.137	1.131	0.837	No
CAN GTI vs. TCAN GTI	1.153	1.068	0.817	No
CDBAN GTI vs. BDCAN GTI	1.058	0.979	0.798	No
IAN GTI vs. TBAN GTI	0.975	0.903	0.748	No
TCAN GTI vs. DCAN GTI	0.517	0.479	0.865	No
TBAN GTI vs. DBAN GTI	0.162	0.161	0.872	No

***N*-Acetylcysteine thiol reactivity analyses.** The *N*-acetylcysteine (NAC) thiol reactivity high throughput assay is a screen to identify potential adverse biological effects.[17-19] The cysteine thiol is a reductant against reactive toxicants.[20, 21] HANs were reacted with NAC for 20 min in a volume of 50 μL, followed by the addition of 50 μL of 5,5-dithiobis (2-nitrobenzoic acid) (DTNB, 1 mM). Each experiment included concurrent negative controls (Tris buffer and NAC), positive controls (Tris buffer, NAC and maleimide), and corresponding blanks to correct for the background A_{412}. After incubation with NAC (20 min, 30 °C shaking, dark conditions), DTNB was added to quantify the available thiol groups. The microplate was analyzed at 412 nm using a Molecular Devices Spectramax Paradigm multimode microplate reader after linear shaking of 10 s. The data were saved in an Excel spreadsheet. The A_{412} values for each well were blank-corrected. The blank-corrected negative controls were averaged. This value was divided into the individual A_{412} values for each treatment group ×100; the data were expressed as the percent of the concurrent negative controls. Using these normalized data, we generated concentration-response curves. Regression analyses were used to calculate the EC_{50} values, the effective HAN concentration that induced a reduction in the NAC thiol response by 50% compared to the concurrent negative controls.

The concentration-response graphs illustrating the NAC thiol reactivity of the HANs are presented in Figures S21 to S30.

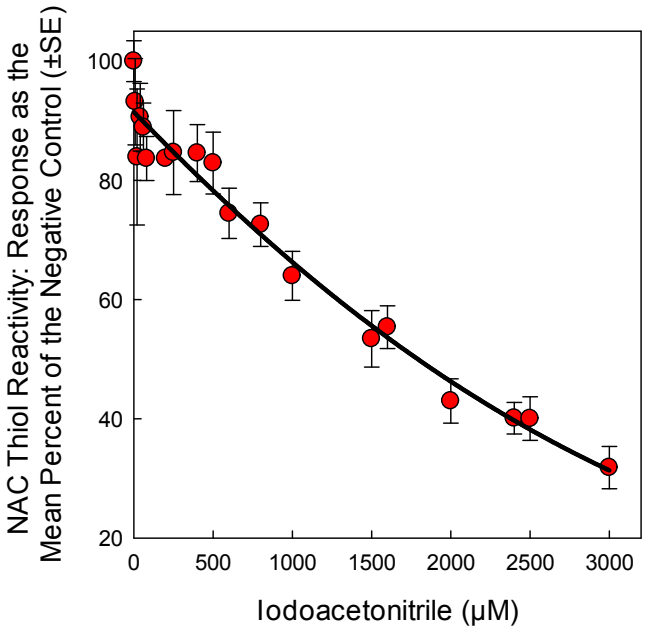

Figure S21. NAC thiol reactivity concentration-response curve for IAN. Mean (±SE) EC_{50} value was 1714.47 ± 70.15 μM.

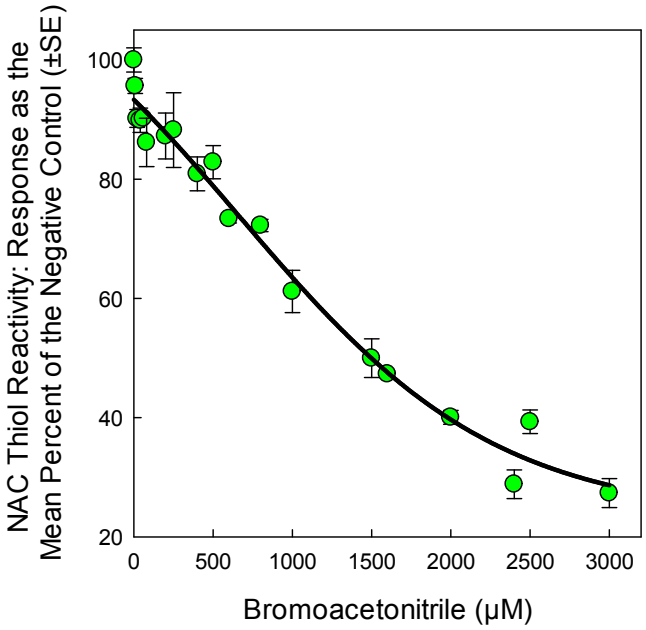

Figure S22. NAC thiol reactivity concentration-response curve for BAN. Mean (±SE) EC_{50} value was 1502.96 ± 16.88 μM.

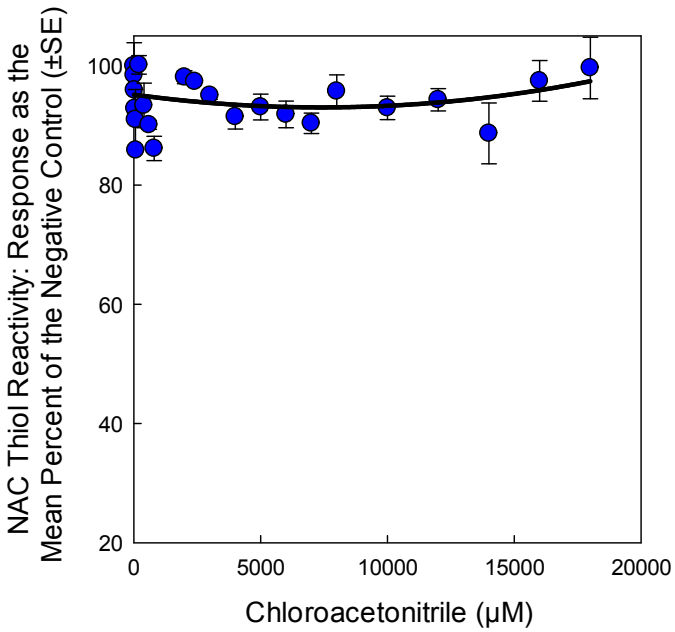

Figure S23. NAC thiol reactivity concentration-response curve for CAN.

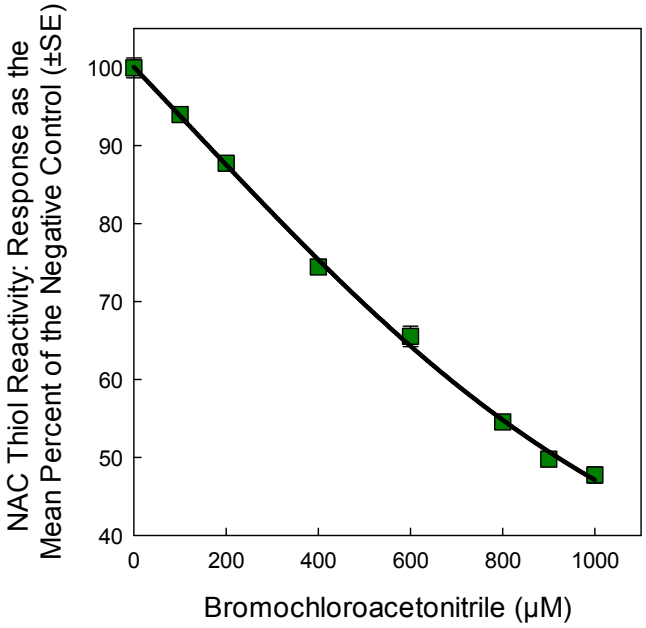

Figure S24. NAC thiol reactivity concentration-response curve for BCAN. Mean (±SE) EC_{50} value was 912.84 ± 11.11 µM.

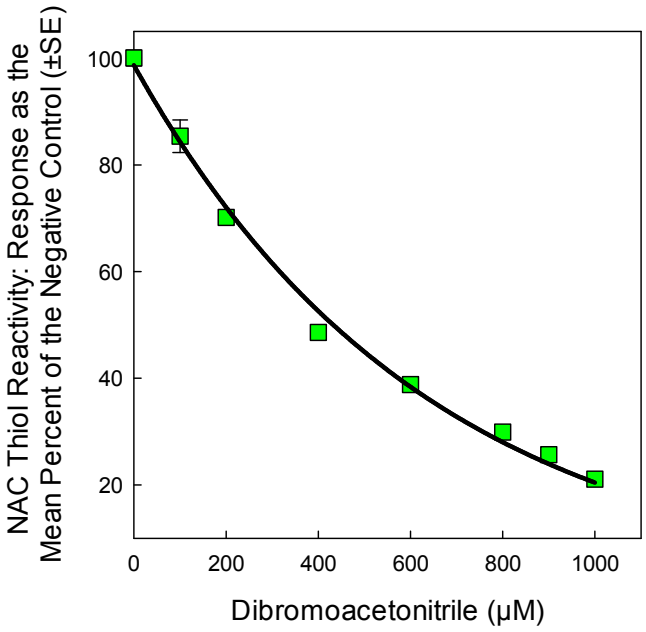

Figure S25. NAC thiol reactivity concentration-response curve for DBAN. Mean (±SE) EC_{50} value was 403.78 ± 3.92 µM.

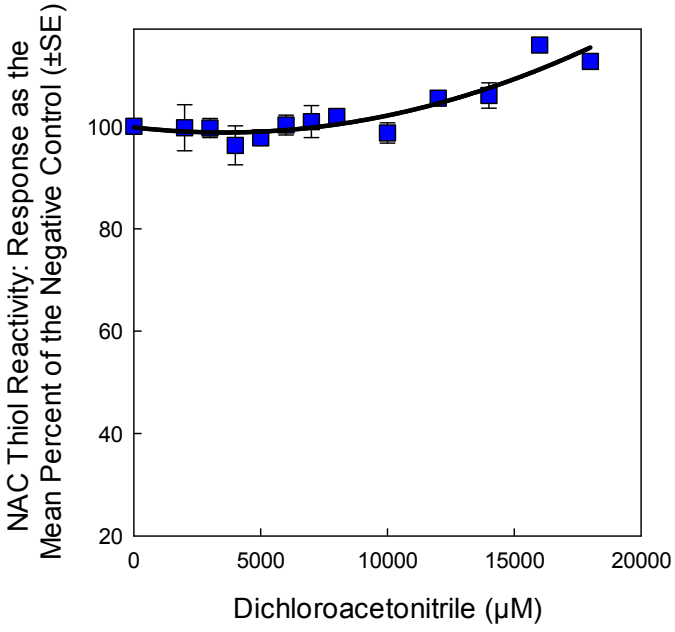

Figure S26. NAC thiol reactivity concentration-response curve for DCAN.

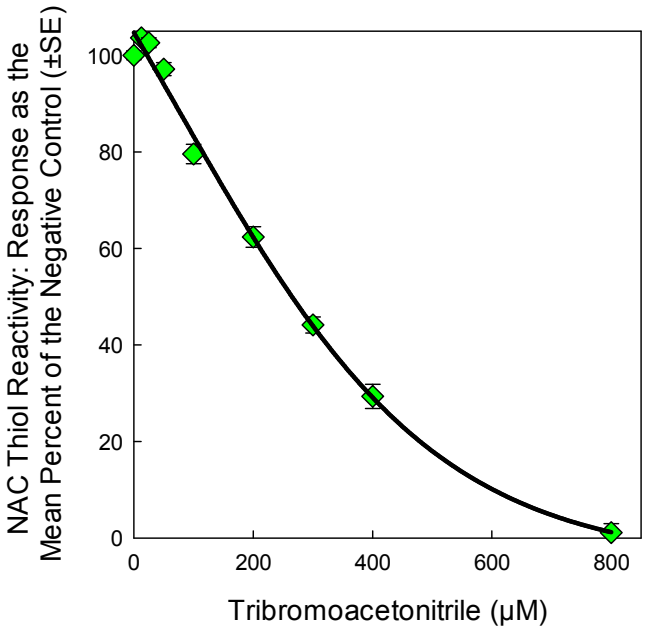

Figure S27. NAC thiol reactivity concentration-response curve for TBAN. Mean (±SE) EC_{50} value was 263.06 ± 6.73 µM.

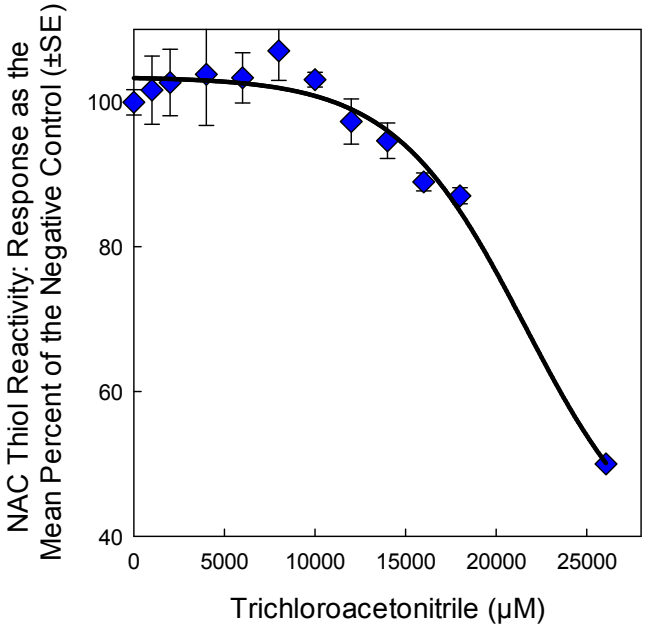

Figure S28. NAC thiol reactivity concentration-response curve for TCAN. The extrapolated mean (±SE) EC_{50} value was 26148.67 ± 9.83 µM.

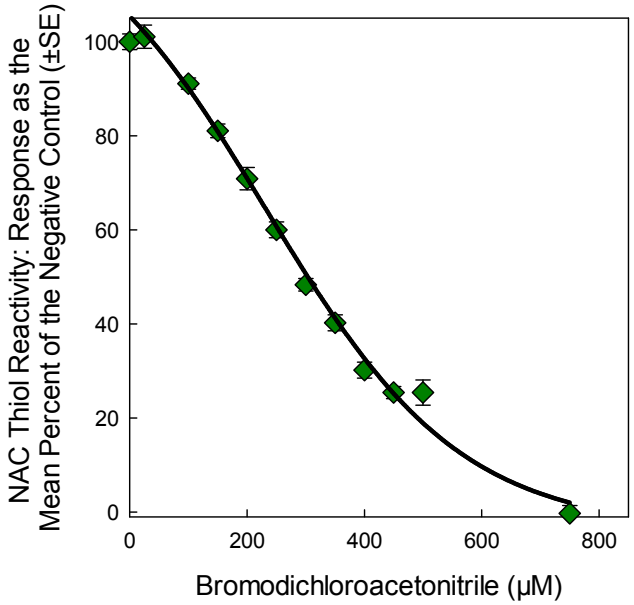

Figure S29. NAC thiol reactivity concentration-response curve for BDCAN. The extrapolated mean (±SE) EC_{50} value was 302.31 ± 2.04 µM.

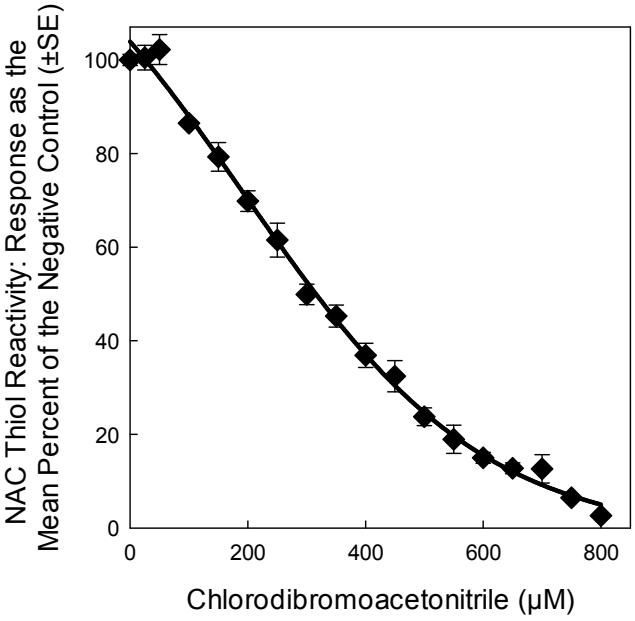

Figure S30. NAC thiol reactivity concentration-response curve for CDBAN. The extrapolated mean (±SE) EC_{50} value was 314.43 ± 5.74 µM.

Table S17. One Way Analysis of Variance: Iodoacetonitrile (IAN) thiol reactivity response as the percent of the negative control.

Group Name	N	Missing	Mean	Std Dev	SEM
0 IAN	5	0	100.000	0.000	0.000
8 IAN	5	0	94.640	7.924	3.544
20 IAN	5	0	81.632	12.495	5.588
40 IAN	5	0	89.466	6.206	2.775
60 IAN	5	0	88.077	4.471	1.999
80 IAN	5	0	82.926	4.048	1.810
200 IAN	5	0	83.655	0.000	0.000
250 IAN	5	0	89.427	10.813	4.836
400 IAN	5	0	85.529	5.233	2.340
500 IAN	5	0	84.905	7.103	3.176
600 IAN	5	0	75.314	4.610	2.062
800 IAN	5	0	73.314	3.998	1.788
1000 IAN	5	0	60.759	6.692	2.993
1500 IAN	5	0	55.306	6.387	2.856
1600 IAN	5	0	54.652	3.937	1.761
2000 IAN	5	0	41.919	5.755	2.574
2400 IAN	5	0	40.621	2.903	1.298
2500 IAN	5	0	37.108	6.007	2.686
3000 IAN	5	0	32.876	4.591	2.053

Source of Variation	DF	SS	MS	F	P
Between Groups	18	41021.732	2278.985	59.679	<0.001
Residual	76	2902.250	38.187		
Total	94	43923.982			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001). Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 IAN vs. 3000 IAN	67.124	17.175	<0.001	Yes
0 IAN vs. 2500 IAN	62.892	16.092	<0.001	Yes
0 IAN vs. 2400 IAN	59.379	15.193	<0.001	Yes
0 IAN vs. 2000 IAN	58.081	14.861	<0.001	Yes
0 IAN vs. 1600 IAN	45.348	11.603	<0.001	Yes
0 IAN vs. 1500 IAN	44.694	11.436	<0.001	Yes
0 IAN vs. 1000 IAN	39.241	10.040	<0.001	Yes
0 IAN vs. 800 IAN	26.686	6.828	<0.001	Yes
0 IAN vs. 600 IAN	24.686	6.316	<0.001	Yes
0 IAN vs. 20 IAN	18.368	4.700	<0.001	Yes
0 IAN vs. 80 IAN	17.074	4.369	<0.001	Yes
0 IAN vs. 200 IAN	16.345	4.182	<0.001	Yes
0 IAN vs. 500 IAN	15.095	3.862	0.001	Yes
0 IAN vs. 400 IAN	14.471	3.703	0.002	Yes
0 IAN vs. 60 IAN	11.923	3.051	0.013	Yes
0 IAN vs. 250 IAN	10.573	2.705	0.025	Yes
0 IAN vs. 40 IAN	10.534	2.695	0.017	Yes
0 IAN vs. 8 IAN	5.360	1.372	0.174	No

Table S18. One Way Analysis of Variance: Bromoacetonitrile (BAN) thiol reactivity response as the percent of the negative control.

Group Name	N	Missing	Mean	Std Dev	SEM
0 BAN	6	0	99.997	4.971	2.029
8 BAN	2	0	95.643	1.776	1.256
20 BAN	2	0	90.184	2.104	1.488
40 BAN	2	0	89.884	2.883	2.039
60 BAN	2	0	90.217	0.608	0.430
80 BAN	2	0	86.169	5.746	4.063
200 BAN	2	0	87.246	5.438	3.845
250 BAN	3	0	88.225	10.845	6.261
400 BAN	2	0	80.894	4.024	2.845
500 BAN	4	0	82.856	5.575	2.787
600 BAN	2	0	73.367	1.004	0.710
800 BAN	2	0	72.242	1.448	1.024
1000 BAN	4	0	61.187	7.089	3.544
1500 BAN	3	0	49.959	5.613	3.241
1600 BAN	2	0	47.309	0.717	0.507
2000 BAN	4	0	40.048	2.345	1.173
2400 BAN	2	0	28.826	3.395	2.401
2500 BAN	3	0	39.303	3.419	1.974
3000 BAN	4	0	27.345	4.842	2.421

Source of Variation	DF	SS	MS	F	P
Between Groups	18	31768.426	1764.913	67.714	<0.001
Residual	34	886.188	26.064		
Total	52	32654.614			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001). Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 BAN vs. 3000 BAN	72.652	22.046	<0.001	Yes
0 BAN vs. 2000 BAN	59.950	18.191	<0.001	Yes
0 BAN vs. 2400 BAN	71.171	17.074	<0.001	Yes
0 BAN vs. 2500 BAN	60.695	16.813	<0.001	Yes
0 BAN vs. 1500 BAN	50.038	13.861	<0.001	Yes
0 BAN vs. 1600 BAN	52.688	12.640	<0.001	Yes
0 BAN vs. 1000 BAN	38.810	11.777	<0.001	Yes
0 BAN vs. 800 BAN	27.756	6.658	<0.001	Yes
0 BAN vs. 600 BAN	26.630	6.388	<0.001	Yes
0 BAN vs. 500 BAN	17.141	5.201	<0.001	Yes
0 BAN vs. 400 BAN	19.103	4.583	<0.001	Yes
0 BAN vs. 80 BAN	13.828	3.317	0.015	Yes
0 BAN vs. 250 BAN	11.772	3.261	0.015	Yes
0 BAN vs. 200 BAN	12.751	3.059	0.021	Yes
0 BAN vs. 40 BAN	10.113	2.426	0.080	No
0 BAN vs. 20 BAN	9.814	2.354	0.072	No
0 BAN vs. 60 BAN	9.780	2.346	0.049	Yes
0 BAN vs. 8 BAN	4.355	1.045	0.304	No

Table S19. One Way Analysis of Variance: Chloroacetonitrile (CAN) thiol reactivity response as the percent of the negative control.

Group Name	N	Missing	Mean	Std Dev	SEM
0 CAN	8	0	99.984	2.240	0.792
8 CAN	2	0	98.432	7.681	5.431
20 CAN	2	0	95.940	0.529	0.374
40 CAN	2	0	92.835	1.087	0.768
60 CAN	2	0	90.987	7.137	5.047
80 CAN	2	0	85.841	0.374	0.265
200 CAN	2	0	100.156	2.188	1.547
400 CAN	2	0	93.359	5.294	3.744
600 CAN	2	0	90.099	1.050	0.742
800 CAN	2	0	86.153	2.886	2.040
1600 CAN	2	0	107.954	0.000	0.000
2000 CAN	3	0	98.094	1.896	1.095
2400 CAN	2	0	97.362	0.000	0.000
3000 CAN	3	0	95.029	1.138	0.657
4000 CAN	3	0	91.461	3.579	2.066
5000 CAN	3	0	93.095	3.739	2.159
6000 CAN	3	0	91.853	3.864	2.231
7000 CAN	3	0	90.364	2.971	1.715
8000 CAN	6	0	95.747	6.696	2.734
10000 CAN	3	0	92.945	3.402	1.964
12000 CAN	3	0	94.295	3.245	1.874
14000 CAN	3	0	88.672	8.823	5.094
16000 CAN	3	0	97.471	5.899	3.406
18000 CAN	3	0	99.639	8.949	5.167

Source of Variation	DF	SS	MS	F	P
Between Groups	23	1434.989	62.391	2.944	<0.001
Residual	45	953.614	21.191		
Total	68	2388.602			

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

NOTE No concentration response observed. Interpretation, no significant effect on NAC thiol reactivity observed.

Power of performed test with alpha = 0.050: 0.947

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 CAN vs. 80 CAN	14.142	3.886	0.008	Yes
0 CAN vs. 800 CAN	13.831	3.800	0.009	Yes
0 CAN vs. 14000 CAN	11.311	3.630	0.015	Yes
0 CAN vs. 7000 CAN	9.620	3.087	0.067	No
0 CAN vs. 4000 CAN	8.523	2.735	0.156	No
0 CAN vs. 600 CAN	9.885	2.716	0.155	No
0 CAN vs. 6000 CAN	8.131	2.609	0.190	No

0 CAN vs. 60 CAN	8.997	2.472	0.243	No
0 CAN vs. 10000 CAN	7.039	2.258	0.355	No
0 CAN vs. 5000 CAN	6.888	2.210	0.368	No
0 CAN vs. 1600 CAN	7.971	2.190	0.360	No
0 CAN vs. 40 CAN	7.149	1.964	0.497	No
0 CAN vs. 12000 CAN	5.688	1.825	0.574	No
0 CAN vs. 400 CAN	6.624	1.820	0.543	No
0 CAN vs. 8000 CAN	4.237	1.704	0.594	No
0 CAN vs. 3000 CAN	4.954	1.590	0.637	No
0 CAN vs. 20 CAN	4.044	1.111	0.892	No
0 CAN vs. 16000 CAN	2.513	0.806	0.964	No
0 CAN vs. 2400 CAN	2.621	0.720	0.960	No
0 CAN vs. 2000 CAN	1.890	0.606	0.958	No
0 CAN vs. 8 CAN	1.552	0.426	0.965	No
0 CAN vs. 18000 CAN	0.345	0.111	0.992	No
0 CAN vs. 200 CAN	0.172	0.0473	0.962	No

Table S20. One Way Analysis of Variance: Bromochloroacetonitrile (BCAN) thiol reactivity response as the percent of the negative control.

Group Name	**N**	**Missing**	**Mean**	**Std Dev**	**SEM**
0 BCAN	4	0	99.971	2.548	1.274
100 BCAN	3	0	93.972	0.298	0.172
200 BCAN	3	0	87.731	1.383	0.798
400 BCAN	3	0	74.443	1.368	0.790
600 BCAN	3	0	65.519	2.260	1.305
800 BCAN	3	0	54.553	1.575	0.909
900 BCAN	3	0	49.780	1.559	0.900
1000 BCAN	3	0	47.750	1.782	1.029

Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
Between Groups	7	9604.957	1372.137	435.145	<0.001
Residual	17	53.606	3.153		
Total	24	9658.562			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
0 BCAN vs. 1000 BCAN	52.222	38.504	<0.001	Yes
0 BCAN vs. 900 BCAN	50.192	37.008	<0.001	Yes
0 BCAN vs. 800 BCAN	45.418	33.488	<0.001	Yes
0 BCAN vs. 600 BCAN	34.453	25.403	<0.001	Yes
0 BCAN vs. 400 BCAN	25.528	18.823	<0.001	Yes
0 BCAN vs. 200 BCAN	12.241	9.025	<0.001	Yes
0 BCAN vs. 100 BCAN	6.000	4.424	<0.001	Yes

Table S21. One Way Analysis of Variance: Dibromoacetonitrile (DBAN) thiol reactivity response as the percent of the negative control.

Group Name	N	Missing	Mean	Std Dev	SEM
0 DBAN	4	0	100.040	2.949	1.475
100 DBAN	3	0	85.378	5.280	3.048
200 DBAN	3	0	70.173	1.418	0.819
400 DBAN	3	0	48.588	1.382	0.798
600 DBAN	3	0	38.819	0.293	0.169
800 DBAN	3	0	29.912	1.548	0.894
900 DBAN	3	0	25.677	1.000	0.578
1000 DBAN	3	0	21.082	0.818	0.472

Source of Variation	DF	SS	MS	F	P
Between Groups	7	20391.347	2913.050	505.400	<0.001
Residual	17	97.986	5.764		
Total	24	20489.333			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001). Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 DBAN vs. 1000 DBAN	78.959	43.061	<0.001	Yes
0 DBAN vs. 900 DBAN	74.363	40.555	<0.001	Yes
0 DBAN vs. 800 DBAN	70.129	38.245	<0.001	Yes
0 DBAN vs. 600 DBAN	61.222	33.388	<0.001	Yes
0 DBAN vs. 400 DBAN	51.452	28.060	<0.001	Yes
0 DBAN vs. 200 DBAN	29.868	16.289	<0.001	Yes
0 DBAN vs. 100 DBAN	14.662	7.996	<0.001	Yes

Table S22. One Way Analysis of Variance: Dichloroacetonitrile (DCAN) thiol reactivity response as the percent of the negative control.

Group Name	N	Missing	Mean	Std Dev	SEM
0 DCAN	9	0	100.024	10.540	3.513
2000 DCAN	3	0	99.751	7.770	4.486
3000 DCAN	3	0	99.700	3.220	1.859
4000 DCAN	3	0	96.311	6.572	3.794
5000 DCAN	3	0	97.769	2.245	1.296
6000 DCAN	3	0	100.286	3.357	1.938
7000 DCAN	3	0	100.949	5.423	3.131
8000 DCAN	6	0	101.971	2.518	1.028
10000 DCAN	3	0	98.737	3.435	1.983
12000 DCAN	3	0	105.525	2.006	1.158
14000 DCAN	3	0	106.053	4.293	2.479
16000 DCAN	4	0	110.178	11.588	5.794
18000 DCAN	4	0	105.787	13.892	6.946

Source of Variation	DF	SS	MS	F	P
Between Groups	12	670.924	55.910	0.903	0.552
Residual	37	2290.002	61.892		
Total	49	2960.926			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.552).

Table S23. One Way Analysis of Variance: Tribromoacetonitrile (TBAN) thiol reactivity response as the percent of the negative control.

Group Name	N	Missing	Mean	Std Dev	SEM
0 TBAN	6	0	100.018	2.250	0.918
12.5 TBAN	3	0	103.624	1.432	0.827
25 TBAN	6	0	102.615	2.502	1.021
50 TBAN	6	0	97.162	3.320	1.355
100 TBAN	6	0	79.562	4.924	2.010
200 TBAN	6	0	62.387	5.214	2.129
300 TBAN	3	0	44.157	2.911	1.680
400 TBAN	6	0	29.352	6.130	2.502
800 TBAN	6	0	1.121	4.588	1.873

Source of Variation	DF	SS	MS	F	P
Between Groups	8	60741.593	7592.699	433.527	<0.001
Residual	39	683.038	17.514		
Total	47	61424.631			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 TBAN vs. 800 TBAN	98.897	40.931	<0.001	Yes
0 TBAN vs. 400 TBAN	70.666	29.247	<0.001	Yes
0 TBAN vs. 300 TBAN	55.860	18.877	<0.001	Yes
0 TBAN vs. 200 TBAN	37.631	15.574	<0.001	Yes
0 TBAN vs. 100 TBAN	20.456	8.466	<0.001	Yes
0 TBAN vs. 12.5 TBAN	3.606	1.219	0.544	No
0 TBAN vs. 50 TBAN	2.856	1.182	0.429	No
0 TBAN vs. 25 TBAN	2.597	1.075	0.289	No

Table S24. One Way Analysis of Variance: Trichloroacetonitrile (TCAN) thiol reactivity response as the percent of the negative control.

Group Name	N	Missing	Mean	Std Dev	SEM
0 TCAN	3	0	99.944	3.038	1.754
1000 TCAN	3	0	101.627	8.160	4.711
2000 TCAN	3	0	102.675	7.949	4.589
4000 TCAN	3	0	103.806	12.212	7.051
6000 TCAN	3	0	103.325	6.008	3.469
8000 TCAN	3	0	107.048	7.076	4.086
10000 TCAN	3	0	103.071	1.775	1.025
12000 TCAN	3	0	97.290	5.395	3.115
14000 TCAN	3	0	94.639	4.244	2.450
16000 TCAN	3	0	88.948	2.162	1.248
18000 TCAN	3	0	84.348	0.706	0.408

Source of Variation	DF	SS	MS	F	P
Between Groups	10	1444.913	144.491	3.698	0.005
Residual	22	859.490	39.068		
Total	32	2304.403			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.005).

Power of performed test with alpha = 0.050: 0.860

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 TCAN vs. 18000 TCAN	15.597	3.056	0.050	Yes
0 TCAN vs. 16000 TCAN	10.996	2.155	0.323	No
0 TCAN vs. 8000 TCAN	7.103	1.392	0.791	No
0 TCAN vs. 14000 TCAN	5.306	1.040	0.925	No
0 TCAN vs. 4000 TCAN	3.861	0.757	0.974	No
0 TCAN vs. 6000 TCAN	3.381	0.662	0.973	No
0 TCAN vs. 10000 TCAN	3.127	0.613	0.958	No
0 TCAN vs. 2000 TCAN	2.730	0.535	0.935	No
0 TCAN vs. 12000 TCAN	2.655	0.520	0.846	No
0 TCAN vs. 1000 TCAN	1.683	0.330	0.745	No

Table S25. One Way Analysis of Variance: Bromodichloroacetonitrile (BDCAN) thiol reactivity response as the percent of the negative control.

Group Name	N	Missing	Mean	Std Dev	SEM
BDCAN 0	14	0	99.989	6.243	1.668
25 BDCAN	3	0	101.056	4.277	2.469
50 BDCAN	3	0	105.776	1.052	0.607
100 BDCAN	3	0	91.096	2.062	1.190
150 BDCAN	3	0	81.060	2.504	1.446
200 BDCAN	3	0	70.891	4.122	2.380
250 BDCAN	3	0	60.022	2.913	1.682
300 BDCAN	3	0	48.350	2.309	1.333
350 BDCAN	3	0	40.242	2.941	1.698
400 BDCAN	3	0	30.183	2.925	1.689
450 BDCAN	3	0	25.416	2.218	1.280
500 BDCAN	3	0	25.409	4.644	2.681
750 BDCAN	3	0	-0.266	2.882	1.664

Source of Variation	DF	SS	MS	F	P
Between Groups	12	56808.208	4734.017	239.250	<0.001
Residual	37	732.117	19.787		
Total	49	57540.325			

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

Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
BDCAN 0 vs. 750 BDCAN	100.255	35.426	<0.001	Yes
BDCAN 0 vs. 500 BDCAN	74.580	26.353	<0.001	Yes
BDCAN 0 vs. 450 BDCAN	74.573	26.351	<0.001	Yes
BDCAN 0 vs. 400 BDCAN	69.806	24.666	<0.001	Yes
BDCAN 0 vs. 350 BDCAN	59.747	21.112	<0.001	Yes
BDCAN 0 vs. 300 BDCAN	51.639	18.247	<0.001	Yes
BDCAN 0 vs. 250 BDCAN	39.967	14.123	<0.001	Yes
BDCAN 0 vs. 200 BDCAN	29.098	10.282	<0.001	Yes
BDCAN 0 vs. 150 BDCAN	18.929	6.689	<0.001	Yes
BDCAN 0 vs. 100 BDCAN	8.893	3.142	0.010	Yes
BDCAN 0 vs. 50 BDCAN	5.787	2.045	0.094	No
BDCAN 0 vs. 25 BDCAN	1.067	0.377	0.708	No

Table S26. One Way Analysis of Variance: Chlorodibromoacetonitrile (CDBAN) thiol reactivity response as the percent of the negative control.

Group Name	N	Missing	Mean	Std Dev	SEM
0 CDBAN	14	0	99.980	4.465	1.193
25 CDBAN	4	0	100.473	5.246	2.623
50 CDBAN	4	0	102.187	6.387	3.193
100 CDBAN	4	0	86.473	1.304	0.652
150 CDBAN	4	0	79.284	6.125	3.063
200 CDBAN	4	0	69.843	4.396	2.198
250 CDBAN	4	0	61.516	7.247	3.623
300 CDBAN	4	0	49.923	4.354	2.177
350 CDBAN	4	0	45.266	4.734	2.367
400 CDBAN	4	0	36.870	5.150	2.575
450 CDBAN	4	0	32.426	6.679	3.339
500 CDBAN	4	0	23.777	3.786	1.893
550 CDBAN	4	0	18.951	6.014	3.007
600 CDBAN	4	0	14.995	2.339	1.170
650 CDBAN	4	0	12.761	2.335	1.167
700 CDBAN	4	0	12.632	6.065	3.032
750 CDBAN	4	0	6.454	0.188	0.0939
800 CDBAN	3	0	2.652	0.107	0.0618

Source of Variation	DF	SS	MS	F	P
Between Groups	17	104161.331	6127.137	270.028	<0.001
Residual	63	1429.517	22.691		
Total	80	105590.847			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001). Power of performed test with alpha = 0.050: 1.000

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

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 CDBAN vs. 750 CDBAN	93.526	34.631	<0.001	Yes
0 CDBAN vs. 700 CDBAN	87.348	32.344	<0.001	Yes
0 CDBAN vs. 650 CDBAN	87.220	32.296	<0.001	Yes
0 CDBAN vs. 800 CDBAN	97.328	32.115	<0.001	Yes
0 CDBAN vs. 600 CDBAN	84.986	31.469	<0.001	Yes
0 CDBAN vs. 550 CDBAN	81.029	30.004	<0.001	Yes
0 CDBAN vs. 500 CDBAN	76.203	28.217	<0.001	Yes
0 CDBAN vs. 450 CDBAN	67.554	25.014	<0.001	Yes
0 CDBAN vs. 400 CDBAN	63.110	23.369	<0.001	Yes
0 CDBAN vs. 350 CDBAN	54.714	20.260	<0.001	Yes
0 CDBAN vs. 300 CDBAN	50.058	18.535	<0.001	Yes
0 CDBAN vs. 250 CDBAN	38.464	14.243	<0.001	Yes
0 CDBAN vs. 200 CDBAN	30.138	11.159	<0.001	Yes
0 CDBAN vs. 150 CDBAN	20.696	7.663	<0.001	Yes
0 CDBAN vs. 100 CDBAN	13.507	5.002	<0.001	Yes
0 CDBAN vs. 50 CDBAN	2.207	0.817	0.660	No
0 CDBAN vs. 25 CDBAN	0.493	0.182	0.856	No

Table S27. One Way Analysis of Variance: HAN TRI Comparisons.

Group Name	N	Missing	Mean	Std Dev	SEM
IAN TRI	6	0	0.589	0.0631	0.0258
BAN TRI	6	0	0.666	0.0186	0.00761
DBAN TRI	6	0	2.478	0.0587	0.0240
BCAN TRI	6	0	1.096	0.0326	0.0133
TBAN TRI	9	0	3.820	0.279	0.0931
TCAN TRI	6	0	0.0382	0.000214	0.0000875
BDCAN TRI	11	0	3.309	0.0745	0.0225
CDBAN TRI	11	0	3.190	0.183	0.0553

Source of Variation	DF	SS	MS	F	P
Between Groups	7	113.463	16.209	809.632	<0.001
Residual	53	1.061	0.0200		
Total	60	114.524			

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

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
TBAN TRI vs. TCAN TRI	3.782	50.717	<0.001	Yes
BDCAN TRI vs. TCAN TRI	3.271	45.553	<0.001	Yes
CDBAN TRI vs. TCAN TRI	3.152	43.896	<0.001	Yes
TBAN TRI vs. IAN TRI	3.232	43.338	<0.001	Yes
TBAN TRI vs. BAN TRI	3.155	42.302	<0.001	Yes
BDCAN TRI vs. IAN TRI	2.721	37.890	<0.001	Yes
BDCAN TRI vs. BAN TRI	2.644	36.814	<0.001	Yes
TBAN TRI vs. BCAN TRI	2.724	36.529	<0.001	Yes
CDBAN TRI vs. IAN TRI	2.602	36.233	<0.001	Yes
CDBAN TRI vs. BAN TRI	2.525	35.157	<0.001	Yes
BDCAN TRI vs. BCAN TRI	2.213	30.819	<0.001	Yes
DBAN TRI vs. TCAN TRI	2.440	29.863	<0.001	Yes
CDBAN TRI vs. BCAN TRI	2.094	29.162	<0.001	Yes
DBAN TRI vs. IAN TRI	1.889	23.126	<0.001	Yes
DBAN TRI vs. BAN TRI	1.812	22.181	<0.001	Yes
TBAN TRI vs. DBAN TRI	1.343	18.005	<0.001	Yes
DBAN TRI vs. BCAN TRI	1.381	16.911	<0.001	Yes
BCAN TRI vs. TCAN TRI	1.058	12.952	<0.001	Yes
BDCAN TRI vs. DBAN TRI	0.832	11.581	<0.001	Yes
CDBAN TRI vs. DBAN TRI	0.713	9.925	<0.001	Yes
TBAN TRI vs. CDBAN TRI	0.630	9.906	<0.001	Yes
TBAN TRI vs. BDCAN TRI	0.511	8.035	<0.001	Yes
BAN TRI vs. TCAN TRI	0.628	7.682	<0.001	Yes
IAN TRI vs. TCAN TRI	0.550	6.736	<0.001	Yes
BCAN TRI vs. IAN TRI	0.508	6.216	<0.001	Yes
BCAN TRI vs. BAN TRI	0.431	5.270	<0.001	Yes
BDCAN TRI vs. CDBAN TRI	0.119	1.972	0.105	No
BAN TRI vs. IAN TRI	0.0773	0.946	0.349	No

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