

1 **Toxicity of Haloacetonitrile Mixtures to a Normal Tissue-Derived Human Cell**
2 **Line: Are they additive, synergistic, or antagonistic?**

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17 **ABSTRACT**

18 Haloacetonitriles (HANs)—a class of nitrogen-containing disinfection by-products found in
19 treated drinking water—are cytotoxic and genotoxic to mammalian cells. However, most cell
20 toxicity data have been ascertained using transformed animal or cancer-derived human cell lines.
21 In this study, we evaluated the cytotoxicity of individual chloro-, bromo-, and iodo-acetonitrile
22 (ClCH₂CN, BrCH₂CN, and ICH₂CN) and their mixtures using normal tissue-derived human
23 epithelium-derived RPE-1^{hTERT} cells. The order for individual HAN cytotoxicity from most to
24 least toxic was ICH₂CN>BrCH₂CN>>ClCH₂CN with the inhibitory concentration that reduced

25 the cell viability by 50% of the untreated cells (IC_{50}) of $2.52 \pm 0.19 \mu M$, 7.24 ± 0.68 , and 190 ± 18.5
26 μM , respectively. For HAN mixtures cytotoxicity from most to least toxic was
27 $BrCH_2CN + ICH_2CN > ICH_2CN + ClCH_2CN \approx$
28 $ClCH_2CN + BrCH_2CN + ICH_2CN > ClCH_2CN + BrCH_2CN$ with a total IC_{50} of 4.65 ± 0.71 , 8.12 ± 1 ,
29 7.91 ± 0.64 and $13.6 \pm 2.04 \mu M$, respectively. The cytotoxicity of all four mixtures at IC_{50} were
30 well predicted by both concentration addition (CA) and independent action (IA) models, which
31 confirmed additivity effects. However, the Chou-Talalay method (CT) showed antagonistic
32 cytotoxic effects. The difference could primarily stem from the different threshold criteria of
33 each model for additivity, synergy and antagonism, leading to different conclusions. Results
34 indicate that evaluating cumulative mixture toxic effects with CA, IA and CT can improve the
35 overall confidence of the analysis.

36 **Keywords:** Chou-Talalay method, disinfection byproducts, haloacetonitriles, RPE-1^{hTERT} cells,
37 mixture toxicity, cytotoxicity

38

39 **Synopsis:** Results show that the combined cytotoxic effects of monohaloacetonitrile mixtures
40 were additive.

41

42 INTRODUCTION

43 Chemical disinfectants and UV light may react with organic matter and other components in
44 source waters to unintentionally form disinfection by-products (DBPs).¹⁻³ Additionally, source
45 waters with nitrogen-containing compounds may react with disinfectants to form nitrogen-
46 containing DBPs.⁴⁻¹³ Haloacetonitriles (HANs) are among of the most frequently detected and

47 abundant class of DBPs^{8,13}—up to a HAN total of 41 µg/L in drinking water—and were
48 identified as major drivers for overall toxicity in disinfected drinking waters.¹⁴ Although HANs
49 are not regulated, dichloroacetonitrile and dibromoacetonitrile were included in the Fifth
50 Contaminant Candidate List' by the U.S. Environmental Protection Agency.¹⁵

51 The toxicity of individual HANs has been studied extensively *in vitro*. HANs are reported to
52 be 1-2 orders of magnitude more cytotoxic and genotoxic to Chinese Hamster Ovary (CHO) cells
53 relative to regulated DBPs.^{16,17} Comparable HAN cytotoxicity outcomes have also been reported
54 in human-derived hepatocellular carcinoma (HepG2) cells,¹⁸ protozoa (*T. pyriformis*), bacteria
55 (*V. fischeri*, *S. typhimurium*), and budding yeast (*S. cerevisiae*).¹⁹⁻²¹ However, characterization of
56 mixed exposure effects of HANs is important because, in practice, humans are exposed to DBP
57 as mixtures, and rarely (if ever) as single agents.^{22,23} To predict overall water toxicity, several
58 studies have used the largest database of *in vitro* DBP toxicity data based on CHO cells¹⁷ and
59 computed toxicity values based on the assumption that combined effects of DBP mixtures are
60 additive.^{6,9,11,24-26} A recent study showed that the assumption is valid for the cytotoxicity of CHO
61 cells; however, these cells may differ substantially from human cells.²⁷ Additive toxicity was also
62 observed for 38 DBPs tested using reporter gene assays in one human cell line and one bacterial
63 assay.²⁸ HANs were observed to dominate the overall water toxicity of these mixtures^{27,28};
64 however, mixtures of di- and tri-HANs have shown antagonistic effects in a human cancer cell
65 line.¹⁸ These contradicting results indicate the need for further systematic and well controlled
66 study, particularly for HAN mixtures and using a normal tissue-derived human cell line, which
67 might better predict their impact on the health of human cells.

68 Different models have been used to evaluate combined toxic effects of DBP mixtures. The
69 most commonly used methods in environmental studies are concentration addition (CA), and

70 independent action (IA).²⁸⁻³³ However, these models assume that mixture components do not
71 interact and their mixture effects can be added where components have the same mode of action
72 (CA), or different sites of action (IA). The Chou-Talalay method (CT) is a widely used analysis
73 in the biomedical field³⁴⁻³⁷ to evaluate the effects of combining two or more compounds and has
74 gained recent interest in DBP mixture studies.^{29,38-40} The CT dose-effect analysis is performed by
75 fitting scattering data points to a *median-effect equation* based on the mass-action law as further
76 explained in Text S1 in Supporting Information (SI). The *median-effect equation* is a generalized
77 equation that relates dose and effect independent of number of components, products or
78 mechanisms derived from hundreds of mechanistically distinct equations in biomedical science
79 and does not use any statistical method or assumption.^{34,35} Lasch *et al.* evaluated virtual data sets
80 with these three models and identified mixed results with all of them; however, they observed an
81 overall better prediction with CT but recommended the use of CA and IA in parallel to increase
82 the confidence of the analysis.⁴¹

83 Given the knowledge gap concerning the effects of HAN mixtures in a normal tissue-derived
84 human cell line, the objectives of this study were to (1) characterize the cytotoxicity of individual
85 monoHANs and their mixtures to a normal tissue-derived human cell line, (2) determine the
86 combined cytotoxicity of monoHANs using CA, IA and CT. Identifying the type of toxic
87 interactions among unregulated HANs may help improve the safety of treated water.

88

89 **METHODS AND MATERIALS**

90 **Reagents and solutions.** Reagents and solution preparation are detailed in Text S2 in SI.

91 **Cell culture.** Human cell line RPE-1^{hTERT} was obtained from ATCC (#CRL-4000) and
92 maintained in DMEM: F12 (Gibco #11330-032) containing 10 % (v/v) fetal bovine serum and

93 0.01 mg/mL Hygromycin B (Sigma, #10687010) at 37°C and 5% CO₂ in a humidified
94 atmosphere. Cells were subcultured every two days and were used for no more than 24 passages.

95 **Cytotoxicity.** The reduction of cell viability was measured using the alamarBlue cell
96 viability assay. The alamarBlue assay measures the reducing environment of the living cell and
97 may signify an impairment of cellular metabolism.⁴² The protocol used in this study was adapted
98 from the Thermofisher's alamarBlue assay protocol (alamarBlue® Assay-U.S. Patent No.
99 5,501,959) with slight modifications as described in Text S3 in SI. Each experiment had six
100 internal replicates and was repeated on three different days. All combinations and individual
101 cytotoxicity testing were performed on the same day to minimize variations. Cell viability was
102 calculated as the mean percentage of the negative control of each experiment. Statistical analysis
103 of the data is described in Text S4 in SI. Cell growth was also monitored throughout the
104 treatment and described in Text S5 in SI.

105 **Cytotoxicity of HAN mixtures.** To evaluate the combined toxicity effects of HAN
106 mixtures, binary and tertiary combinations of chloroacetonitrile (ClCH₂CN), bromoacetonitrile
107 (BrCH₂CN), and iodoacetonitrile (ICH₂CN) were prepared by mixing monoHANs in equal
108 concentrations (1:1 and 1:1:1). The total concentration of HAN mixtures ranged between 0.1-100
109 μM. Dose-response curves from HAN mixtures were analyzed with CA,^{28,43-44} IA,⁴⁵ and the
110 CT^{34-37,46} and further explained in Text S1 in SI. The accuracy of each model was evaluated with
111 the model deviation ratio (MDR)^{28,32,41} for CA and IA and the combination index (CI) for CT.³⁵⁻³⁷
112 MDR is defined as

113
$$MDR = \frac{\text{Measured Mixture Values}}{\text{Predicted Mixture Values}}$$

114 where, the measured value for CA is the mixture concentration that affects $x\%$ of the population
115 and the predicted value is calculated with $IC_{x,mix}$ (Text S1 in SI). For IA, the numerator is the
116 measured total effect for a given mixture at specific concentrations and the predicted values is
117 calculated with $I(C_{mix})$ (Text S1 in SI). A value between $0.5 \leq MDR \leq 2$ has a deviation within a
118 factor of 2 and has an acceptable fit to the model.³² The MDR used in this study is inverted to
119 indicate synergism for $MDR < 0.5$ and antagonism for $MDR > 2$.⁴¹

120 CI calculates the combined effects of a mixture at different concentrations and response
121 levels using dose-response curves for individual compounds and is defined as

$$122 \quad CI = \sum_{i=1}^n \frac{d_i}{D_i}$$

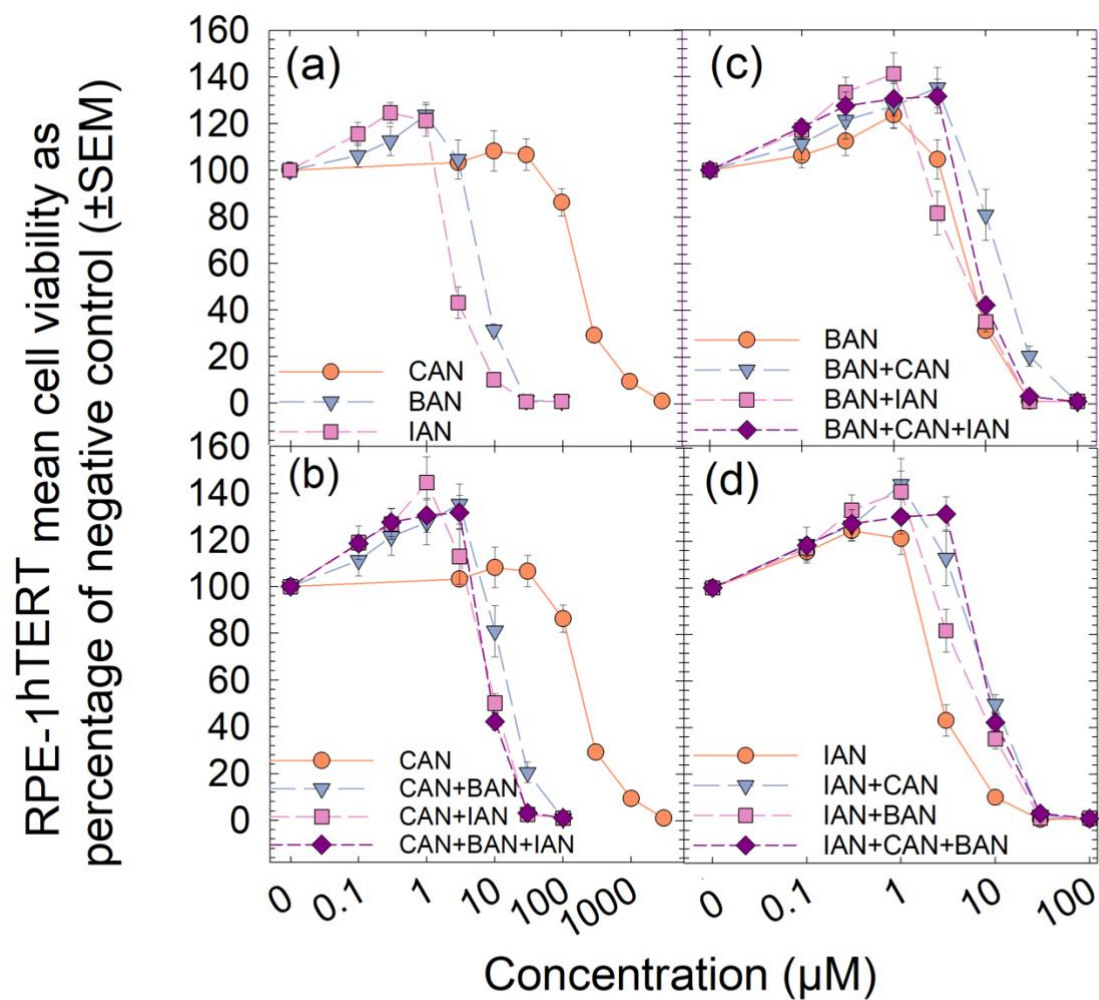
123 where, d_i is the actual dose of mixture component i at which a specific adverse effect is induced
124 as a mixture. D_i is the calculated dose of the i component at which the same effect is induced
125 individually (Text S1 in SI). The threshold for additivity, synergism, and antagonism are
126 $0.9 \leq CI \leq 1.1$, $CI < 0.9$, and $CI > 1.1$, respectively.³⁵⁻³⁷

127

128 RESULTS AND DISCUSSION

129 **Cytotoxic effects of individual HANs.** MonoHANs were previously reported by our
130 research group to be stable in cell culture media for three days at 37°C and 5% CO₂,⁴⁷ whereas
131 the di- and tri-substituted HANs degrade rapidly within this same time frame. To minimize
132 confounding effects from HAN degradation products, RPE-1^{hTERT} cells were exposed to
133 monoHANs and evaluated for cytotoxicity. RPE-1^{hTERT} cells were used because they are a
134 normal-tissue derived and have a stable genome with a model chromosome number of 46, which
135 makes them a widely-used epithelial cell model to study DNA damage.⁴⁸ Furthermore, DBP

136 exposure has been linked to bladder cancer,^{49,50} an epithelial-derived cancer. Dose-response
137 curves for ClCH₂CN, BrCH₂CN, and ICH₂CN are shown in Figure 1a. Cell viability was reduced
138 in a dose-dependent manner, where ClCH₂CN was the least potent, followed by BrCH₂CN and
139 ICH₂CN as the most potent. The calculated inhibitory concentration that reduced cell viability by
140 50% (IC₅₀) for ClCH₂CN, BrCH₂CN, and ICH₂CN were 190±18.5, 7.24±0.68, and 2.52±0.19
141 μM, respectively. The order of monoHAN potency observed in this study agrees with those
142 reported in CHO cells,¹⁶ HepG2 cells,¹⁸ *T. pyriformis*, *V. fischeri*, and *S. cerevisiae*.¹⁹⁻²¹



143

144 **Figure 1:** Reduction of RPE-1^{hTERT} cell viability with increasing concentrations of mono-
 145 HANs and their binary (molar ratio of 1:1) and tertiary (molar ratio of 1:1:1) combinations.
 146 (a)Cytotoxicity of monoHANs (b)ClCH₂CN (CAN) and their mixtures, (c)BrCH₂CN (BAN)
 147 and their mixtures and (d)ICH₂CN (IAN) and their mixtures.

148

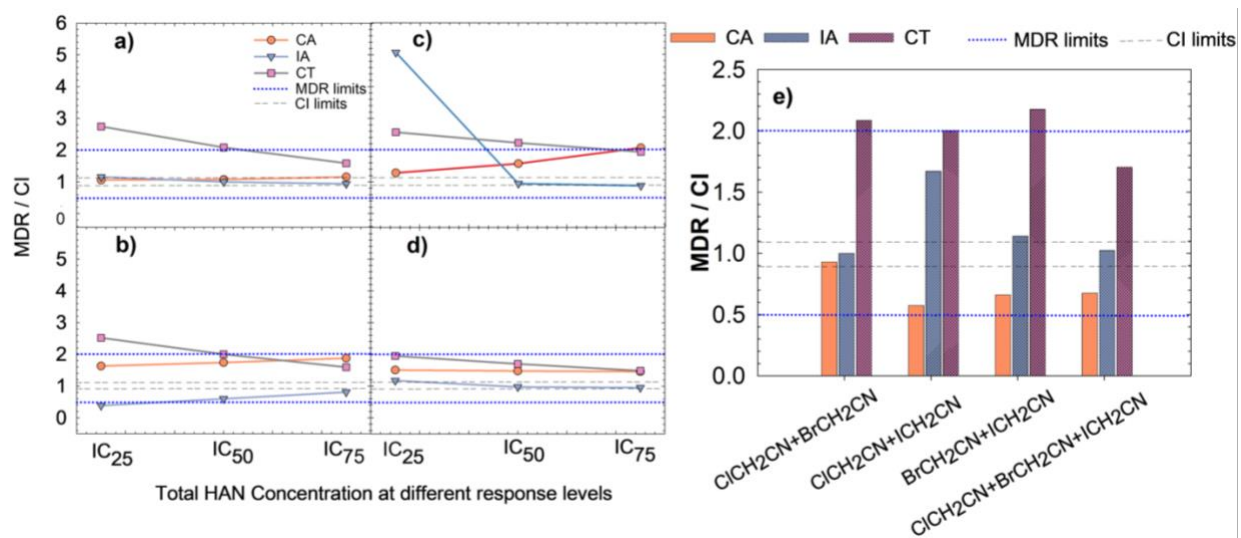
149 While the alamarBlue assay measures viable cell capacity to reduce resazurin (non-
150 fluorescent and blue color) to resorufin and dihydroresorufin (fluorescent and red color), it does
151 not measure cell growth.⁵¹ For that reason, the impact of HANs on cell growth was evaluated
152 separately (Figure S1 in SI). Cell growth rate was impacted for all compounds and at all
153 concentrations tested, in a dose-dependent manner, with the highest two concentrations showing
154 robust cytostatic and cytotoxic effects.-These results corroborate the effects observed for the
155 alamarBlue assay.

156

157 **Cytotoxic effect of HAN mixtures.** In disinfected waters, HANs exist as mixtures.
158 Therefore, the combined effect of binary and tertiary mixtures of HANs on a human cell line was
159 evaluated between 0.1-100 μM —a range of concentrations for which RPE-1^{hTERT} cells are
160 viable at individual exposures (Figure 1a). Dose-response curves for each monoHAN are shown
161 in Figure 1a and each is presented together for comparison with its respective mixtures in Figure
162 1b-d. IC_{50} values for mixtures (1:1 and 1:1:1) $\text{ClCH}_2\text{CN}+\text{BrCH}_2\text{CN}$, $\text{ClCH}_2\text{CN}+\text{ICH}_2\text{CN}$,
163 $\text{BrCH}_2\text{CN}+\text{ICH}_2\text{CN}$, and $\text{ClCH}_2\text{CN}+\text{BrCH}_2\text{CN}+\text{ICH}_2\text{CN}$ were 13.6 ± 2.04 , 8.12 ± 1 , 4.65 ± 0.71
164 and 7.91 ± 0.64 μM , respectively (Figure S2 in SI). For BrCH_2CN (Figure 1c), the addition of
165 ClCH_2CN attenuated the toxicity of BrCH_2CN while the mixture with ICH_2CN had the opposite
166 effect. Mixtures of ICH_2CN (Figure 1d) with any other monoHAN attenuated the toxicity of
167 ICH_2CN . However, mixtures with ClCH_2CN (Figure 1b) were markedly more toxic than
168 ClCH_2CN alone, but only at concentrations well beyond what is found in disinfected waters.^{8,13}
169 This reflects the impact of compounds with greater potency (BrCH_2CN and ICH_2CN) on one
170 with lesser potency (ClCH_2CN). Furthermore, the $\text{ClCH}_2\text{CN}+\text{BrCH}_2\text{CN}+\text{ICH}_2\text{CN}$ mixture was
171 observed to have a similar IC_{50} to $\text{BrCH}_2\text{CN}+\text{ICH}_2\text{CN}$ mixture. RPE-1^{hTERT} cells treated with

172 HAN mixtures (Figure S1d-g in SI) also exhibited similar cell growth/inhibition patterns as the
 173 RPE-1^{hTRET} cells treated with individual monoHANs (Figure S1a-c in SI) except for the 10 μ M
 174 ClCH₂CN+BrCH₂CN mixture, which slowed cell growth but did not result in cell death.

175 The combined cytotoxicity effect of the monoHAN mixtures was evaluated with CA, IA and
 176 CT models (Figures 2 and S3-S4 in SI). The MDR/CI compares the deviation of the
 177 experimental data with each model shown in Figure 2. Results for ClCH₂CN+BrCH₂CN (Figure
 178 2a and S3a in SI) agree with both CA and IA for IC₂₅-IC₇₅. Results for ClCH₂CN+ICH₂CN
 179 (Figures 2b and S3b in SI) agree with CA for IC₂₅-IC₇₅ and IA between IC₅₀-IC₇₅. Synergistic
 180 effects were observed for IA at IC₂₅. In contrast, BrCH₂CN+ICH₂CN (Figures 2c and S3c in SI)
 181 agree with both CA between IC₂₅-IC₅₀ and IA between IC₅₀-IC₇₅ and were antagonistic for both
 182 IA at IC₂₅ and CA at IC₇₅ concentrations. Results for ClCH₂CN+BrCH₂CN+ICH₂CN (Figures
 183 2d and S3d in SI) also agree with both CA and IA for IC₂₅-IC₇₅. The cytotoxicity of all four
 184 mixtures at IC₅₀ agree with both CA and IA at IC₅₀ (Figure 2e).



185
 186 **Figure 2.** Model deviation ratio (MDR) or combination index (CI) values for concentration
 187 addition (CA), independent action (IA), or Chou-Talalay (CT) model of

188 (a)ClCH₂CN+BrCH₂CN, (b)ClCH₂CN+ICH₂CN, (c)BrCH₂CN+ICH₂CN,
189 (d)ClCH₂CN+BrCH₂CN+ICH₂CN, and (e)all mixtures at IC₅₀. The limits for
190 additivity are represented by the dotted blue lines for CA and IA and the black dashed
191 lines for CT.

192 These results indicate that the effects on cell viability from different modes of action could
193 be additive and, therefore, agree with both models. Escher *et al.* found that for mixtures at low-
194 level effects (<30%), CA and IA predictions are similar up to 10% of the effect, suggesting that
195 IA effects are additive.³¹ Pals *et al.* showed that HANs are highly reactive to cellular thiols,
196 including glutathione and N-acetyl L-cysteine, which are important cellular antioxidants.⁵²
197 Recently, Yueng *et al.* observed that HANs react with human proteome thiols in live cells to
198 form adducts.⁵³ Therefore, it is possible that HANs can interact with different molecular sites to
199 have an overall additive effect, which might explain why HAN mixtures in this study agree with
200 both CA and IA models.

201 For CT, all four mixtures had CI values greater than 1 for <IC₉₀ or fraction of cells
202 affected (f_a) lower than 0.9 (Figures 2 and S4, Table S1 in SI). These results indicate that HAN
203 mixture concentrations up to $f_a \leq 0.90$ have antagonistic effects on cell growth or viability.
204 Additionally, it can be observed (Figure S4 in SI) that with increasing HAN concentration the
205 curve approached $CI \rightarrow 1$, which indicates that the combined effects of HANs shift from strong
206 antagonism to nearly additive. However, these combined effects occur at low and high HAN
207 concentrations, which indicate that either the alamarBlue assay is not sensitive enough to detect
208 changes in cell population or the cell population is almost all dead. Moreover, it was observed
209 that as the mixture combinations changed from binary to tertiary, the interaction of HANs for a

210 given percent effect tended to approach additivity. Although these results require further study
211 and confirmation with mixtures with higher number of DBPs or at potency ratios, it agrees with
212 previous studies that have observed an additive combined effect of multicomponent DBP
213 mixtures.^{27,31}

214 CT results differ from CA and IA and could be partially because of the limitations of the CA
215 and IA models, which assume analytes do not interact with each other. Furthermore, the range
216 considered to be “additive” between CA/IA models and CT do not necessarily agree, particularly
217 because there is no standardized criteria for additivity, synergism, and antagonism.^{36,41} For that
218 reason, it is important to evaluate the combined effects of mixtures using all three models to
219 obtain a higher degree of confidence in the analysis.⁴¹

220 In general, the data are well predicted by both CA and IA except at concentrations that
221 exhibit antagonism, which agrees with CT. Results from this study also agree with Lu *et al.* who
222 measured and evaluated the toxicity of binary and tertiary mixtures of dichloro-, dibromo-, and
223 trichloro-acetonitrile with the CA and IA models.¹⁸ The dichloro- and dibromo-acetonitrile
224 mixture agreed with both IA and CA, while all other mixtures with trichloroacetonitrile showed
225 antagonistic effects. Dawson *et al.* also found that binary combinations of ClCH₂CN, BrCH₂CN,
226 and ICH₂CN (1:1.22 potency ratios) exposed to *V. fischeri* were well predicted by both CA and
227 IA, with MDR values between 0.79-1.11.²⁰

228

229 **IMPLICATIONS**

230 To our knowledge this is the first study that has systematically evaluated the individual and
231 combined cytotoxic effects of monoHANs in a normal tissue-derived human cell model. In
232 general, the CA and IA models showed that the combined effect of mixtures is additive.
233 However, according to CT, binary and tertiary mixtures of monoHANs act antagonistically when
234 present together. The differences could be related to the assumption that components of the
235 mixture do not interact with each other (CA and IA models) and the criteria for additivity,
236 synergy and antagonism of each model are not the same. Analyzing cumulative toxic effects of
237 mixtures using CA, IA, and CT can increase the confidence of the analysis and should be
238 considered in future mixture studies. This study showed that monoHANs present as mixtures
239 have additive toxicity that should be taken into consideration when estimating overall toxicity of
240 disinfected waters.

241 **Conflict of interest**

242 The authors declare NO competing (financial or non-financial) interests, or other conflicts of any
243 kind.

244 **Supporting Information:** detailed description of models used to evaluate DBP mixture toxicity;
245 description of reagents and solutions, alamarBlue assay, statistical analysis, and cell growth
246 analysis; figure on cell growth results; figure of haloacetonitrile concentration that reduced cell
247 viability by 50%; figure of experimental and predicted monoHAN combined toxicity effect with
248 CA and IA; figure of CI versus fraction of cells affected for monoHAN mixtures; table with the
249 type of interaction of mixture components according to CI values and references.

250 **Associated Content**

251 Thilina K. Jayawardana; Aaron A. Goodarzi; Ebba U. Kurz; Tatek Temesgen; Susana Y. Kimura;
252 Toxicity of Haloacetonitrile Mixtures to a Normal Tissue-Derived Human Cell Line: Are they
253 additive, synergistic, or antagonistic? 2024, ChemRxiv.
254 <https://chemrxiv.org/engage/chemrxiv/article-details/6700766151558a15ef1955c4> (accessed
255 April 9, 2025).

256 **Acknowledgment**

257 The authors want to thank the following University of Calgary staff and students: Drs. Dustin
258 Pearson and Daniel Berger for training in cell culture and cytotoxicity assays; Drs. Jurgen Gailer,
259 Anne Vaahtokari, Peter Brownlee, and Justin Pals for valuable discussion and advice; John
260 Danforth and Dr. Luc Provencher for their training, discussion, and support, especially during the
261 COVID-19 pandemic restrictions. A special thanks to Shilpa Salgia for her help with purchasing
262 and coordinating our access to instrumentation and other facilities. Funding was provided by the
263 Natural Sciences and Engineering Research Council (NSERC) Discovery Grant, University of
264 Calgary, Canada Research Chair, Canada Foundation for Innovation, and the Robson DNA
265 Science Centre.

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