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

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## ABSTRACT

Haloacetonitriles (HANs), a class of nitrogen-containing disinfection by-products found in treated drinking water, are cytotoxic and genotoxic to mammalian cells. However, most cell toxicity data has been ascertained using transformed or cancer-derived animal and human cell lines with an ambiguous relevance to human health. In this study, we evaluated the cytotoxicity of individual chloro-, bromo-, and iodo-acetonitrile ($ClCH_2CN$, $BrCH_2CN$, and $ICH_2CN$) and their mixtures using normal tissue-derived human epithelium-derived RPE-$1^{hTERT}$ cells. The order for individual HAN cytotoxicity from most to least toxic was

$ICH_2CN>BrCH_2CN>>ClCH_2CN$ with the inhibitory concentration that reduced the cell viability by 50% of the untreated cells ($IC_{50}$) of 3.0, 8.7, and 219.8 μM, respectively. For HAN mixtures cytotoxicity from most to least toxic was $BrCH_2CN+ICH_2CN>ICH_2CN+ClCH_2CN>ClCH_2CN+BrCH_2CN+ICH_2CN>ClCH_2CN+BrCH_2CN$ with $IC_{50}$ of 8.9, 9.9, 10.1 and 17.8 μM, respectively. The cytotoxicity of $ClCH_2CN+BrCH_2CN$ was well predicted by both concentration addition (CA) and independent action (IA) models. The CA model overestimated the toxicity of the other three mixtures which indicates an antagonistic effect with a model deviation ratio of less than 2. The IA model predicted the cytotoxicity of $BrCH_2CN+ICH_2CN$ and $ClCH_2CN+BrCH_2CN+ICH_2CN$ slightly better than the CA model. According to the Chou-Talalay method, all binary mixtures showed strong antagonistic cytotoxic effects, particularly at low concentrations. However, binary mixtures with $ClCH_2CN$ had a slight synergistic effect at high concentrations.

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

***Synopsis:*** Results show that the combined cytotoxic effects of monohaloacetonitrile mixtures—closer to what people are exposed to—was mostly antagonistic.

## INTRODUCTION

Water disinfection is one of the most important public health advances of the 20th century, significantly reducing waterborne diseases, including typhoid and cholera.[1] However, chemical disinfectants and UV light may react with organic matter and other components in source waters

to unintentionally form disinfection by-products (DBPs).[2,3] Additionally, source waters with nitrogen-containing compounds may react with disinfectants to form nitrogen-containing DBPs (N-DBPs).[4-13] Haloacetonitriles (HANs) are among of the most frequently detected and abundant class of N-DBPs[8,13]—up to a HAN total of 41 µg/L in drinking water—and were identified as major drivers for overall toxicity in disinfected drinking waters.[14] Although HANs are not regulated, the World Health Organization has maximum concentration guidelines for dichloroacetonitrile and dibromoacetonitrile of 20 and 70 µg/L,[15,16] respectively; these were included in the Fifth Contaminant Candidate List' by the U.S. Environmental Protection Agency.[17]

The toxicity of individual HANs has been studied extensively *in vitro*. HANs are reported to be 1-2 orders of magnitude more cytotoxic and genotoxic to Chinese Hamster Ovary (CHO) cells relative to regulated DBPs.[18,19] Comparable HAN cytotoxicity outcomes have also been reported in human-derived hepatocellular carcinoma cells (HepG2),[20] protozoa (*Tetrahymena pyriformis*), bacteria (*Vibrio fischeri*, *Salmonella typhimurium*), and budding yeast (*Saccharomyces cerevisiae*). [21-23] However, characterization of mixed exposure effects of HANs is important because, in practice, humans are exposed to DBP as mixtures, and rarely (if ever) as single agents.[24,25] To predict overall water toxicity, several studies have used the largest database of *in vitro* DBP toxicity data based on CHO cells[19] and computed toxicity values based on the assumption that combined effects of DBP mixtures are additive.[6,9,11,26-28] A recent study showed that the assumption is valid for the cytotoxicity of CHO cells; however, these cells may differ substantially from human cells.[29] Additive toxicity was also observed for 38 DBPs tested using reporter gene assays in one human cell line and one bacterial assay.[30] HANs were observed to dominate the overall water toxicity of these mixtures[29,30]; however, mixtures of di-HANs have

shown antagonistic effects in a human cancer cell line.[20] These contradicting results indicate the need for further systematic and well controlled study, particularly for HAN mixtures and using normal tissue-derived human cell line, which might better predict their impact on the health of human cells.

Different models have been used to evaluate cumulative toxic effects of DBP mixtures. The most commonly used methods in environmental studies are concentration addition (CA), and independent action (IA).[30-34] However, when toxicity is over- or under-estimated, it is difficult to quantify the level of antagonism or synergism. The Chou-Talalay combination index is one of the most commonly used calculations to evaluate the effects of combining two compounds and can address some of the shortcomings of the CA and IA models.[35-37] The Chou-Talalay dose-effect analysis is performed based on the mass-action law-based theory where the "median" is a common link between single and multiple entities.[38] Most recently, studies have applied the Chou-Talalay method to study the combined effects of DBPs on embryos of *Platynereis dumerilii* and bacteria (*E. Coli* and *Vibrio qinghaiensis* sp.-Q67).[31,39,40]

Given the knowledge gap concerning the effects of HAN mixtures in a normal tissue-derived human cell line, the objectives of this study were to (1) characterize the cytotoxicity of individual monoHANs and their mixtures to a normal tissue-derived human cell line, (2) determine the combined cytotoxicity of monoHANs using the Chou-Talalay method and compare it to other commonly used models. Identifying the type of toxic interactions among unregulated HANs may help improve the safety of treated water.

## MATERIAL AND METHODS

**Reagents and solutions.** Reagents and solution preparation are detailed in Text S1 in Support Information (SI).

**Cell culture.** Human cell line RPE-1$^{hTERT}$ was obtained from ATCC (#CRL-4000) and maintained in DMEM: F12 (Gibco #11330–032) containing 10 % (v/v) fetal bovine serum and 0.01 mg/mL Hygromycin B (Sigma, #10687010) at 37˚C and 5% $CO_2$ in a humidified atmosphere. Cells were subcultured every two days and were only used for no more than 24 passages.

**Cytotoxicity**. The reduction of cell viability was measured using the alamarBlue cell viability assay. AlamarBlue assay measures the reducing environment of the living cell and may signify an impairment of cellular metabolism.[41] The protocol used in this study was adopted from the Thermofisher's alamarBlue assay protocol (alamarBlue® Assay-U.S. Patent No. 5,501,959) with slight modifications as described in Text S2 in SI. Each experiment had 6 internal replicates and was repeated in triplicate on 3 different days. Altogether, each treatment had 18 replicates. All combinations and individual cytotoxicity testing were performed on the same day to minimize variations. Cell viability was calculated as the mean percentage of the negative control. Statistical analysis of the data is described in Text S3 in SI. Cell growth was also monitored throughout the treatment and described in Text S4 in SI.

**Cytotoxicity of HAN mixtures**. To evaluate the cumulative toxicity effects of HAN mixtures, binary and tertiary combinations of chloroacetonitrile ($ClCH_2CN$), bromoacetonitrile ($BrCH_2CN$), and iodoacetonitrile ($ICH_2CN$) were prepared by mixing monoHANs in equal concentrations (1:1 and 1:1:1). The total concentration of HAN mixtures ranged between 0.1-100 μM. Dose-response curves from HAN mixtures were analyzed with CA, [30,42,43] IA,[44] model

deviation ratio (MDR),[30,34] and the Chou-Talalay method.[35-37,45] Models are explained in Text S5 in SI.

## RESULTS AND DISCUSSION

**Cytotoxic effects of individual HANs**. MonoHANs were previously reported by our research group to be stable in cell culture media for three days at 37°C and 5% $CO_2$,[46] whereas the di- and tri-substituted HANs degraded rapidly within this same time frame. To minimize confounding effects from HAN degradation products, RPE-$1^{hTERT}$ cells were exposed to monoHANs and evaluated for cytotoxicity. RPE-$1^{hTERT}$ cells were used because they are a normal-tissue derived and have a stable genome with a model chromosome number of 46 that makes them widely-used epithelial cell model to study DNA damage.[47] Dose-response curves for $ClCH_2CN$, $BrCH_2CN$, and $ICH_2CN$ are shown in Figure 1a. Cell viability was reduced in a dose-dependent manner, where $ClCH_2CN$ was the least potent, followed by $BrCH_2CN$ and $ICH_2CN$ as the most potent. The calculated inhibitory concentration that reduced cell viability by 50% ($IC_{50}$) for $ClCH_2CN$, $BrCH_2CN$, and $ICH_2CN$ were 220 µM, 8.68 µM, and 3.00 µM, respectively. The order of monoHAN potency observed in this study agrees with those reported in CHO cells, protozoa (*T. pyriformis)*, bacteria (*V. fischeri*), and yeast (*S. cerevisiae*).[21-23] For CHO cells, $ICH_2CN$ had a similar $IC_{50}$ values (3.30 µM) to our study. However, $ClCH_2CN$ (68.3 µM) and $BrCH_2CN$ (3.21 µM) were more potent in CHO cells than in RPE-$1^{hTERT}$ cells.[18] Another study also found that $BrCH_2CN$ is more potent than $ClCH_2CN$ to HepG2 with $IC_{50}$ values of 7 µM and 734 µM, respectively.[20]

While the alamarBlue assay measures viable cell capacity to reduce resazurin (non-fluorescent and blue color) to resorufin and dihydroresorufin (fluorescent and red color), it does not measure cell growth.[48] For that reason, the impact of HANs on cell growth was evaluated separately(Figure S1 in SI). Cell growth rate was impacted for all compounds and at all concentrations tested, in a dose-dependent manner, with the highest two concentrations showing robust cytostatic and cytotoxic effects.-These results corroborate the effects observed for the alamarBlue assay (Figure 1a-g).

**Cytotoxic effect of HAN mixtures**. In disinfected waters, HANs exist as mixtures. Therefore, the combined effect of binary and tertiary mixtures of HANs on a human cell line was evaluated between 0.1-100 µM —a range of concentrations that RPE-1$^{hTERT}$ cells are viable at individual exposures (Figure 1a). Dose-response curves for each monoHAN are shown in Figure 1a and each is presented together for comparison with its respective mixtures in Figure 1b-d. $IC_{50}$ values for mixtures (1:1 and 1:1:1) $ClCH_2CN$+$BrCH_2CN$, $ClCH_2CN$+$ICH_2CN$, $BrCH_2CN$+$ICH_2CN$, and $ClCH_2CN$+$BrCH_2CN$+$ICH_2CN$ were 17.8, 10.1, 9.0 and 9.91 µM respectively. For $BrCH_2CN$ (Figure 1c) and $ICH_2CN$ (Figure 1d), addition of any other HAN attenuated the toxicity of either monoHAN; this attenuation is greatest when $BrCH_2CN$ or $ClCH_2CN$ is mixed with $ICH_2CN$. However, mixtures with $ClCH_2CN$ (Figure 1b) were markedly more toxic than $ClCH_2CN$ alone, but only at higher concentrations well beyond what is found in disinfected waters.[8,13] This reflects the impact of compounds with greater potency ($BrCH_2CN$ and $ICH_2CN$) on one with lesser potency ($ClCH_2CN$). These observations contradict to a study that reported additive toxic effects in *V. fischeri* from binary mixtures of monoHANs, which could be attributed to the difference in combination ratios applied.[22] Dawson *et al*. used a relative

potency ratio of $ICH_2CN$: $BrCH_2CN$ (1:1.22),[22] whereas in our study we used an equalmolar ratios of (1:1 and 1:1:1). Moreover, the intracellular reaction of HANs with components of different cell medium and metabolites, cellular enzymes and other proteins of different test organisms or cellular models might vary. Further study is needed using equipotent ratios to evaluate the cumulative cytotoxic effect of monoHAN mixtures. RPE-$1^{hTERT}$ cells treated with HAN mixtures (Figure S1d-g in SI) also exhibited similar cell growth/inhibition patterns as the RPE-$1^{hTRET}$ cells treated with individual monoHANs (Figure S1a-c in SI) except for the 10 µM $ClCH_2CN$+$BrCH_2CN$ mixture which slowed cell growth but not cell death.

The combined cytotoxicity effect of the monoHAN mixtures was evaluated with the CA and IA models. Results for $ClCH_2CN$+$BrCH_2CN$ (Figure 2a) agree with the IA and CA models. The MDR compares the deviation of the experimental data with each model and the $IC_{50}$ were calculated to be 1.05 and 1.00 for CA and IA, respectively. These results show that the data are well predicted by both models. However, for mixtures containing $ICH_2CN$ (Figure 2b-d), the CA model overestimates toxicity (most conservative) and the IA model underestimates the experimental toxicity data. The MDR for the CA model for $ClCH_2CN$+$ICH_2CN$, $BrCH_2CN$+$ICH_2CN$, and $ClCH_2CN$+$BrCH_2CN$+$ICH_2CN$ mixtures at $IC_{50}$ were 0.55, 0.52, and 0.66, respectively. These results show that the measured toxicity of the mixture is less toxic than expected and the combined toxic effects were borderline antagonistic and observed in Figure 1. Results from this study also agree with Lu *et al.* who they measured and evaluated the toxicity of binary and tertiary mixtures of dichloro-, dibromo-, and trichloro-acetonitrile with the CA and IA models.[20] The dichloro- and dibromo-acetonitrile mixture had a better match with IA than CA, and all other mixtures with trichloroacetonitrile showed antagonistic effects. However, the MDR at $IC_5$ (low concentration) for all mixtures was between 0.64-1.17 and seem to be well predicted

by the CA model. This result is particularly important because HANs are typically present in disinfected waters at low concentrations with individual maximum concentrations between 0.2-12 µg/L.[8,13] The MDR for the IA model for $ClCH_2CN$+$ICH_2CN$, $BrCH_2CN$+$ICH_2CN$, and $ClCH_2CN$+$BrCH_2CN$+$ICH_2CN$ mixtures at $IC_{50}$ were 2.57, 1.66, and 1.05, respectively. These results suggest that with the exception of $ClCH_2CN$+$ICH_2CN$ the mixtures are reasonably well predicted by the IA model.

CA and IA models have limitations. These models assume that individual components do not interact with each other.[49,50] For the IA model, the assumption that components of the mixture act independently may not be accurate for a large proportion of chemical interactions.[51,52] Also, for the CA model to be valid, dose-response curves should have constant potency ratios (parallel log dose-response curves indicating constant potency ratios).[53,54] In nature, having parallel dose-response curves for each mixture component is infrequent.[55] Furthermore, the CA model requires accurately generated dose-response curves for each component in the mixture, which is impractical for combinations with a larger number of components.[55] In addition, for the IA model to hold, it must be applicable along the entire dose response curve.[54]

The Chou-Talalay method was used to evaluate the combined effects of monoHANs and results were compared with the experimental observations. This method does not require a constant potency ratio among the individual components. Using the potency and the shape of the dose-response curve for individual compounds, the Chou-Talalay method can simulate combined effects at different concentrations and response levels of the chemicals in the mixture. All four mixtures had log(CI) values greater than 0 at $f_a$=0.5 ($IC_{50}$), 0.75 ($IC_{75}$), and 0.9 ($IC_{90}$), as shown in Figure 3. These results indicate that HAN mixture concentrations up to $f_a \leq 0.95$ have

antagonistic effects on cell growth or viability. For example, at $IC_5$ the log(CI) values were between 0.7-1.22, which indicates strong to very strong antagonism. These results differ to the CA model and MDR, which suggested that these mixtures are additive at $IC_5$. This could be partially because of the limitations of the CA model that assumes analytes do not interact with each other and the range considered to be "additive" between the CA and IA models and the Chou-Talalay models do not necessarily overlap. For binary mixtures, it can be observed that with increasing HAN concentration the curve approached log(CI)→0 which indicates that the combined effects of HANs shift from strong antagonism to nearly additive. However, these combined effects occur at high HAN concentrations which are not necessarily environmentally relevant. Moreover, it was observed that as the mixture combinations changed from binary to tertiary, the interaction of HANs for a given percent of effect tended to approach additivity. Although these results require further study and confirmation with mixtures with higher number of DBPs or at potency ratios, it agrees with previous studies that have observed an additive combined effect of multicomponent DBP mixtures.[29,33] Overall, the Chou-Talalay method seems to better portray the combined toxic effects observed in experiments.

## IMPLICATIONS

To our knowledge, this is the first study that has systematically evaluated the individual and combined cytotoxic effects of monoHANs in a normal tissue-derived human cell model. According to the Chou-Talalay method, binary and tertiary mixtures of monoHANs act antagonistically when present together, especially in environmentally relevant, low concentrations (<12 µg/L). However, the CA model and MDR showed that the combined effect of mixtures is additive. The differences could be related to the assumption of the CA model that

mixture analytes do not act with each other and the MDR and CI values of each model do not necessarily overlap. The Chou-Talalay method also suggested that with increasing number of DBPs the combined toxicity approach additivity however, this needs to be tested with mixtures with higher number of DBPs. This study showed that monoHAN present as mixtures have lower toxicity than those reported individually which should be taken into consideration when estimating overall water toxicity of disinfected waters.

## Conflict of interest

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

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**Tables and Figures**

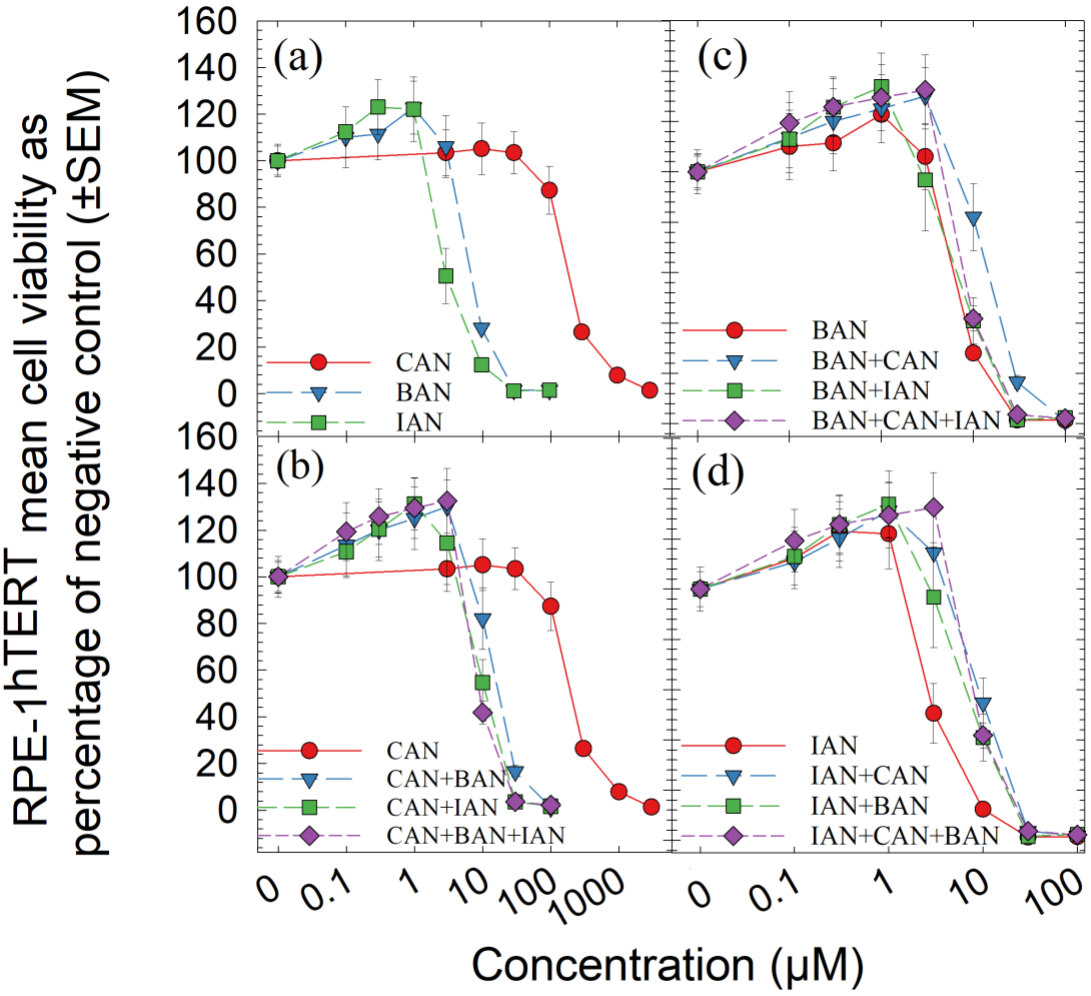


**Figure 1:** Reduction of RPE-1$^{hTERT}$ cell viability with increasing concentrations of mono-HANs and their binary and tertiary combinations. (a) Cytotoxicity of monoHANs to RPE-1 $^{hTERT}$ cells and combined effect of binary (molar ratio of 1:1) and tertiary (molar ratio of 1:1:1) mixtures of monoHANs to RPE-1 cells of (b) $ClCH_2CN$ (CAN) and their mixtures, (c) $BrCH_2CN$ (BAN) and their mixtures and (d) $ICH_2CN$ (IAN) and their mixtures. For Figures a-c 10 µM and above were

statistically different from the negative control ($p < 0.001$). For Figure d the treatments 1 µM and above were statistically different from the negative control ($p < 0.001$).

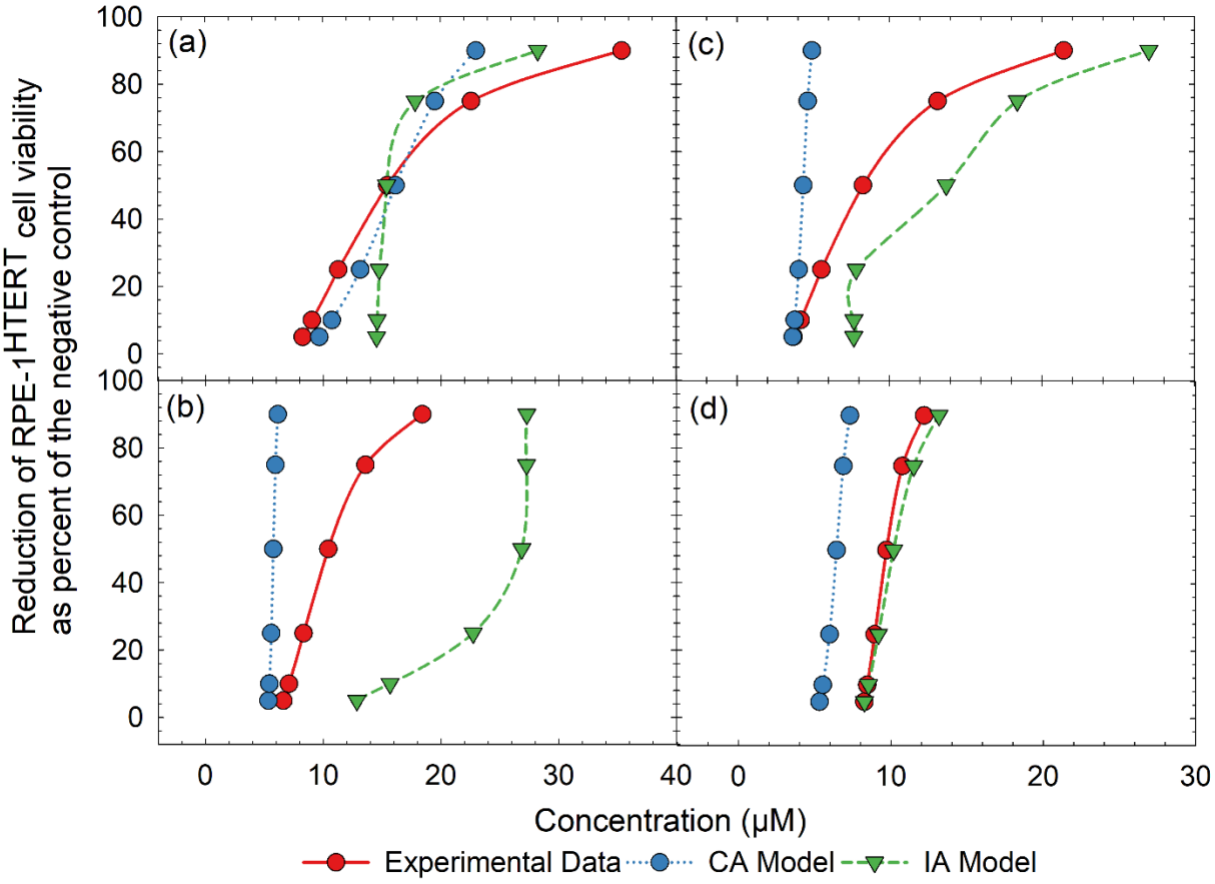


**Figure 2:** Experimental and predicted monohaloacetonitrile cumulative toxicity effect with concentration addition (CA) model and independent action (IA) model of (a) $ClCH_2CN$+$BrCH_2CN$, (b) $ClCH_2CN$+$ICH_2CN$, (c) $BrCH_2CN$+$ICH_2CN$, (d) $ClCH_2CN$+$BrCH_2CN$+$ICH_2CN$

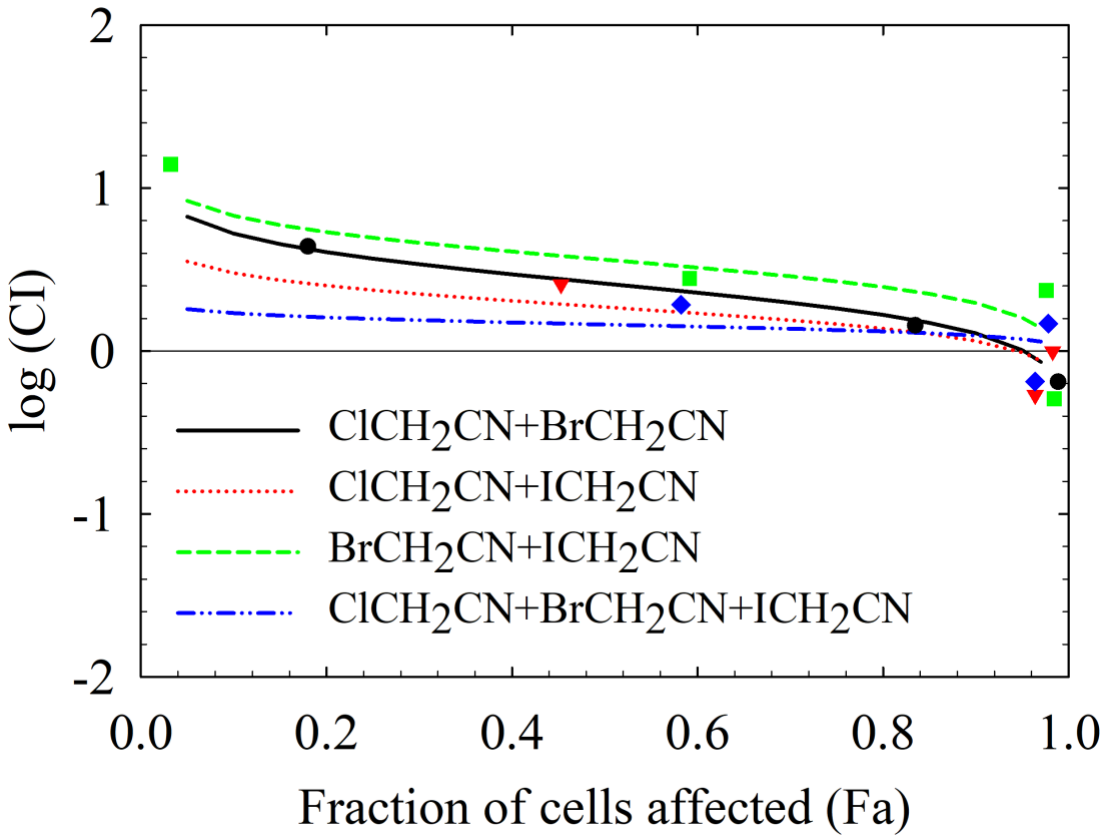


**Figure 3:** The cumulative effect of binary and tertiary combinations of mono-HANs expressed as combination index (CI) versus the fraction of cells affected ($f_a$). C=$ClCH_2CN$, B=$BrCH_2CN$ and I=$ICH_2CN$