Comparative cytotoxic and genotoxic potential of 13 drinking water disinfection by-products using a microplate-based cytotoxicity assay and a developed SOS/umu assay

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Abstract

The implications of disinfection by-products (DBPs) present in drinking water are of public health concern because of their potential mutagenic, carcinogenic and other toxic effects on humans. In this study, we selected 13 main DBPs found in drinking water to quantitatively analyse their cytotoxicity and genotoxicity using a microplate-based cytotoxicity assay and a developed SOS/umu assay in Salmonella typhimurium TA1535/pSK1002. With the developed SOS/umu test, eight DBPs: 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX), dibromoacetonitrile (DBN), iodoacetic acid (IA), bromochloroacetonitrile (BCN), bromoacetic acid (BA), trichloroacetonitrile (TCN), dibromoacetic acid (DBA) and dichloroacetic acid (DCA) were significantly genotoxic to S. typhimurium. Three DBPs: chloroacetic acid (CA), trichloroacetic acid (TCA) and dichloroacetonitrile (DCN) were weakly genotoxic, whereas the remaining DBPs: chloroacetonitrile (CN) and chloral hydrate (CH) were negative. The rank order in decreasing genotoxicity was as follows: MX > DBN > IA > BCN > BA > TCN > DBA > DCA > CA > TCA > DCN > CN > CH. MX was approximately 370 000 times more genotoxic than DCA. In the microplate-based cytotoxicity assay, cytotoxic potencies of the 13 DBPs were compared and ranked in decreasing order as follows: MX > IA > DBN > BA > TCN > DCA > CA > DBA > CH > DCN > CN > DB > BCN > TCA > CH. MX was approximately 19 200 times more cytotoxic than CH. A statistically significant correlation was found between cytotoxicity and genotoxicity of the 13 DBPs in S. typhimurium. Results suggest that microplate-based cytotoxicity assay and the developed SOS/umu assay are feasible tools for analysing the cytotoxicity and genotoxicity of DBPs, particularly for comparing their toxic intensities quantitatively.

Introduction

The first disinfection by-product (DBP) in drinking water was reported in 1974 (1). Since that time, several studies have been directed at assessing whether DBPs pose an adverse health risk. Many DBPs exhibit carcinogenic effects in animals and could be considered as human carcinogens (2–4). To date, more than 600 DBPs have been identified in drinking water (5). Of these, only a small percentage is currently regulated (6). Given the large number of DBPs, many of
them have not yet been quantified and tested for toxicity or carcinogenicity (7). Thus, the most toxicologically significant DBPs should be prioritised for research to compare their toxic potentials.

Genetic toxicology tests are assays performed to detect DNA damage induced by chemical compounds. Such methods are the major regulatory programs worldwide (8). Genetic toxicity data can support evidence that a chemical may cause cancer in animals or humans (9). However, very few assays reported in previous studies have been designed to quantitatively compare the genotoxicity of chemicals, including DBPs. Data for quantitative analysis of the genotoxicity of various DBPs remain limited.

The SOS/\textit{umu} test is a valid test system (ISO standard 13829) for the detection of environmental genotoxicants and potential carcinogens (10,11). A study revealed good agreement between SOS/\textit{umu} (genotoxicity) and Ames test (mutagenicity) results with a concordance of about 90% (12). When the SOS/\textit{umu} test was amended in 1991, it was miniaturised to run in 96-well microplates, which allowed extensive testing of environmental samples (13). In the current study, we developed the SOS/\textit{umu} assay by testing multiple concentrations with multiple replicates. The developed assay was then used to rank the genotoxic effects of 13 DBPs by calculating genotoxicity potency values. The 13 DBPs studied are: six haloacetic acids (HAAs) [chloroacetic acid (CA); dichloroacetic acid (DCA); trichloroacetic acid (TCA); bromoacetic acid (BA); dibromoacetic acid (DBA); iodoacetic acid (IA)], five haloacetonitriles (HANs) [chloroacetonitrile (CN); dichloroacetonitrile (DCN); trichloroacetonitrile (TCN); dibromoacetonitrile (DBN); bromochloroacetonitrile (BCN)], chloral hydrate (CH) and 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone (MX). We selected these 13 chemicals because they constitute the major components of DBPs found in drinking water. Some of these DBPs, such as IA and MX, have shown strong genotoxicity in \textit{Salmonella typhimurium} and mammalian cells (14–17). Except for DCA, TCA, CH and MX, which have been extensively studied, limited genotoxic data are available for the other DBPs. This study aimed to analyse the genotoxicity of the 13 DBPs using the developed SOS/\textit{umu} assay and to determine a relative rank order of genotoxic potency. For comparison, cytotoxicities of the 13 DBPs were also evaluated.

**Materials and Methods**

**Chemicals**

CA, DCA, TCA, BA, DBA, IA, DCN, TCN and DBN were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). CH and MX were obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan). CN and BCN were obtained from J&K Scientific Ltd. (Beijing, China). All other chemical reagents used were of the highest available quality. Prior to use, each DBP was dissolved in pure water with 5% DMSO for future experiments. CAS numbers and purities of the 13 DBPs are presented in Table 1.

**Bacterial strain**

The bacterial strain used in this study was TA1535/pSK1002, an engineered \textit{S. typhimurium} strain transformed with plasmid pSK1002 carrying a fused \textit{umuC}-lacZ gene. This strain was kindly provided by the Tokyo University of Pharmacy and Life Sciences. Laboratory cultures were grown in tryptone glucose ampicillin (TGA) medium at 37°C. The bacteria were maintained at −70°C for long-term storage.

**Microplate-based cytotoxicity assay**

Cytotoxicity assay was performed with \textit{S. typhimurium} TA1535/pSK1002 as described by Pleva et al. (14) with some modifications (18). After the incubation period, the cell titre was adjusted to an optical density (OD) of 0.8 at 600 nm using fresh TGA medium. A 96-well microplate containing a blank control, a negative control and DBP treatment groups was used for the cytotoxicity assay. Briefly, we added 20 µl of 10× TGA medium to each well. To the wells of the blank control and negative control groups was then added 180 µl of solvent (pure water with 5% DMSO). Simultaneously, the DBP treatment groups were added with 180 µl of solvent at different concentrations of DBPs. Finally, the wells of the negative control and DBP treatment groups were seeded with 70 µl of the titrated cells, whereas those of the blank control group were not seeded. The 96-well microplate was incubated for 2h at 37°C at 145 rpm shaking. At the end of the treatment time, 30 µl of the mixture from each well was transferred onto a new 96-well microplate and 270 µl of TGA medium was added. After culturing for 2h, the plate was analysed in a Bio-Rad microplate reader, and the OD was measured at 600 nm. The relative cell density was calculated as follows: \(G = (A_{600, BA} - A_{600, N})/A_{600, N} - A_{600, BA}\), where \(G\) is the relative cell density, \(A_{600, BA}\) is the OD of the sample at 600 nm, \(A_{600, N}\) is the OD of the blank at 600 nm and \(A_{600, N}\) is the OD of the negative control at 600 nm. Regression analysis of the data was conducted to calculate the cytotoxic potency, as well as the concentration of each DBP that reduced the cell density by 50% compared with the negative control.

**Table 1. Characteristics of the 13 DBPs**

<table>
<thead>
<tr>
<th>Chemical class</th>
<th>Chemicals</th>
<th>Abbreviations</th>
<th>CAS No.</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haloacetic acids</td>
<td>Chloroacetic acid</td>
<td>CA</td>
<td>79-11-8</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>Dichloroacetic acid</td>
<td>DCA</td>
<td>79-43-6</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>Trichloroacetic acid</td>
<td>TCA</td>
<td>76-03-9</td>
<td>99.8</td>
</tr>
<tr>
<td></td>
<td>Bromoacetic acid</td>
<td>BA</td>
<td>79-08-3</td>
<td>98.0</td>
</tr>
<tr>
<td></td>
<td>Dibromoacetic acid</td>
<td>DBA</td>
<td>631-64-1</td>
<td>99.9</td>
</tr>
<tr>
<td></td>
<td>Iodoacetic acid</td>
<td>IA</td>
<td>64-69-7</td>
<td>99.5</td>
</tr>
<tr>
<td>Haloacetonitriles</td>
<td>Chloroacetonitrile</td>
<td>CN</td>
<td>107-14-2</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>Dichloroacetonitrile</td>
<td>DCN</td>
<td>3018-12-0</td>
<td>99.5</td>
</tr>
<tr>
<td></td>
<td>Trichloroacetonitrile</td>
<td>TCN</td>
<td>545-06-2</td>
<td>95.0</td>
</tr>
<tr>
<td></td>
<td>Bromochloroacetonitrile</td>
<td>BCN</td>
<td>83463-62-1</td>
<td>87.0</td>
</tr>
<tr>
<td></td>
<td>Dibromoacetonitrile</td>
<td>DBN</td>
<td>3252-43-5</td>
<td>99.9</td>
</tr>
<tr>
<td>Halo furanone</td>
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<td>MX</td>
<td>77419-76-0</td>
<td>98.4</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>Chloral hydrate</td>
<td>CH</td>
<td>75-87-6</td>
<td>99.5</td>
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</table>
SOS/umu assay
The SOS/umu assay was performed according to the procedure of ISO 13829 with slight modifications (19). The first and second plates were prepared as previously described for the cytotoxicity assay. After the incubation of the second plate, we prepared a third plate by pipetting 120 µl of Z-buffer to each well. Up to 120 µl of the mixture from the second plate was transferred onto the third plate column by column. Subsequently, 30 µl of o-nitrophenyl-β-D-galactopyranoside was added to each well. The plate was incubated for 30 min at 30°C at 145 rpm shaking. The reaction was terminated when 120 µl of stop reagent was added to each well. To determine the β-galactosidase activity, the OD was measured at 420 nm. Measurement data were calculated as follows: \( U = \frac{A_{420,S} - A_{420,B}}{A_{600,S} - A_{600,B}} \), where \( U \) is the activity of β-galactosidase, \( A_{420,S} \) is the OD of the sample at 420 nm and \( A_{420,B} \) is the OD of the blank at 420 nm. Genotoxicity assessment is expressed as induction ratios (\( I_R = \frac{U_S}{U_N} \), where \( U_S \) is the activity of β-galactosidase of the sample and \( U_N \) is the activity of β-galactosidase of the negative control. A DBP is considered significantly genotoxic if the maximum induction ratio (\( I_R_{max} \)) ≥ 2.0 and \( G \geq 0.5 \). By contrast, a DBP is considered weakly genotoxic if 2.0 > \( I_R_{max} \) ≥ 1.5 and \( G \geq 0.5 \). To quantitatively compare the DBPs, we defined the genotoxic potency as the concentration at which \( I_R = 2.0 \).

Statistical analyses
Experimental data were recorded on Excel spreadsheets (Microsoft, Inc.) and analysed using TableCurve 2D software (version 5.01, Systat Software, Inc.) and SPSS software (version 11.5, SPSS, Chicago, IL). Dunnett’s test was used to analyse the significant differences between the groups. To evaluate the strength of association between two variables, we used Spearman’s rank correlation analysis. All statistical tests were two-sided with a significance level of 0.05.

Results
Microplate-based cytotoxicity assay
Cytotoxicity assay data were analysed and depicted as a dose-response curve presented in Figure 1. Cell densities decreased with
increasing concentration of DBPs. The two main parameters for cytotoxicity were the lowest cytotoxic concentration and the LC50 value (Table 2). The lowest cytotoxic concentrations among the 13 DBPs ranged from $2.56 \times 10^{-7}$ mol/L (MX) to $2.26 \times 10^{-5}$ mol/L (CH). The order of decreasing LC50 values of the 13 DBPs was MX > IA > DBN > BCN > BA > TCN > DCN > CA > DCA > DBA > CN > TCA > CH. The value of LC50 showed an approximate 1900-fold range from an LC50 value of $3.57 \times 10^{-6}$ mol/L (MX) to $6.87 \times 10^{-5}$ mol/L (CH). IA and DBN were the most cytotoxic DBPs among the six HAAs and five HANs, respectively.

### SOS/umu assay

Genotoxicity assay data are plotted in Figure 2, and curve analysis results are presented in Table 3. Eight DBPs, including MX, IA, DBN, BCN, BA, TCA, TCN, DBA and DCA, exhibited significantly genotoxic effects in *S. typhimurium*. The genotoxic potency was calculated and ranged from $3.27 \times 10^{-8}$ mol/L (MX) to $1.21 \times 10^{-3}$ mol/L (DCA). CA, TCA and DCN were weakly genotoxic with the maximum induction factors between 1.5 and 2.0. CN and CH were the only two DBPs that did not show any genotoxicity. The order of relative genotoxicity was MX > DBN > IA > BCN > BA > TCN > DCA > CA, TCA, DCN > CN, CH. MX was approximately 370 000 times more genotoxic than DCA. The most genotoxic DBPs among the six HAAs and five HANs were IA and DBN, respectively.

### Discussion

The SOS/umu assay is a fast, simple and sensitive bacterial system for the detection of DNA-damaging agents. In this study, all the tested DBPs, except CN and CH, can provoke genotoxic effects (DNA damage) in the SOS/umu test. However, previous in vitro genotoxicity assays on these DBPs produced variable results. The SOS chromotest showed negative results for CA, TCA, BA, CN, DCN and TCN (20,21). Single-cell gel electrophoresis (SCGE, comet) assays revealed that all DBPs, except DCA and TCA, were genotoxic in CHO cells (14,22). All DBPs except CA were also genotoxic in HepG2 cells (23). Therefore, DBP genotoxicity analysis showed a similar sensitivity in the SOS/umu test and the SCGE assay.

However, the SOS/umu test may be more sensitive to DBP genotoxicity than the SOS chromotest. In another SOS/umu assay reported by Ugawa et al. (24) DCA, TCA, DCN and TCN gave negative results for genotoxicity, which is inconsistent with our findings. In our study, the lowest concentration inducing a positive genotoxic response was 15873.02 µmol/L for CA, 13511.09 µmol/L for DCA, 18360.98 µmol/L for TCA and 2021.30 µmol/L for DCN, which are higher than the maximum concentration tested for each compound in the study by Ugawa et al. (24). Therefore, it may be the lower tested doses of CA, DCA, TCA and DCN that account for the negative results in the Ugawa study. The reason of inconsistent results for genotoxicity tests of TCN is not clear. Differences in details in protocols for SOS/umu assay may be part of the reason.

Of the 13 tested DBPs, MX is now widely accepted as a strong mutagen. A previous study indicated that MX exerts up to 67% of the mutagenic activity in extracts of drinking water (25). Further studies confirmed that MX is a multiple-site carcinogen in rodents, with an estimated cancer potency that is 170 times greater than that of chloroform and 17 times greater than that of bromodichloromethane (26). Our results demonstrated that MX is the most potent cytotoxic and genotoxic DBP. A maximum level of MX at 850 ng/L has been observed from a drinking water treatment plant in the USA (27). Therefore, the occurrence of MX in drinking water should be closely monitored, and the risk of MX to humans needs further evaluation.

HAAs represent the largest non-volatile DPB species found in drinking water. The UK Drinking Water Inspectorate recommended that HAAs should be classified as high-priority substances in the list of regulatory chemical parameters to be routinely monitored (28). On the basis of alkylating activity, iodo/bromo-DBPs are expected to be more reactive than their chloro analogues (29). In the current study, IA was the most cytotoxic and genotoxic mono-HAA, followed by BA and CA. The ranking order of IA > BA > CA indicates that the toxic potency of HAAs increases with increasing halogen size. For HAAs, we also observed that increasing halogenation tends to decrease the alkylating activity (cytotoxicity: TCA < DCA < CA, DBA < BA; genotoxicity: TCA < DCA, DBA < BA). These data indicate that the increase in toxic potency is negatively correlated with the degree of halogenation.

### Table 2. Cytotoxicity analysis of 13 DBPs in *S. typhimurium* TA1535/pSK1002

| Chemicals | Concentration range of cytotoxicity assay (mol/L) | Lowest toxic concentration (mol/L) | LC50 value (mol/L) | Toxic rank order | R²
|------------|-----------------------------------------------|----------------------------------|-------------------|-----------------|---
| IA         | 2–108 × 10⁻⁵                                | 5.98 × 10⁻⁵                      | 1.78 × 10⁻⁴       | 2               | 0.97
| BA         | 1–72 × 10⁻⁴                                 | 3.20 × 10⁻⁴                      | 9.59 × 10⁻⁴       | 5               | 0.97
| CA         | 3–318 × 10⁻⁴                                | 1.06 × 10⁻³                      | 1.44 × 10⁻²       | 8               | 0.96
| DCA        | 2–776 × 10⁻⁴                                | 7.76 × 10⁻⁴                      | 1.64 × 10⁻²       | 9               | 0.96
| DBA        | 1–23 × 10⁻⁴                                 | 1.78 × 10⁻²                      | 1.91 × 10⁻²       | 10              | 0.96
| TCA        | 6–918 × 10⁻⁴                                | 2.04 × 10⁻²                      | 4.00 × 10⁻²       | 12              | 0.95
| DBN        | 1–1006 × 10⁻⁴                               | 1.12 × 10⁻⁴                      | 2.00 × 10⁻⁴       | 3               | 0.98
| BCN        | 4–1943 × 10⁻⁴                               | 7.20 × 10⁻⁴                      | 5.15 × 10⁻⁴       | 4               | 0.95
| TCN        | 2–1386 × 10⁻⁴                               | 2.60 × 10⁻⁴                      | 1.35 × 10⁻³       | 6               | 0.98
| DCN        | 3–910 × 10⁻⁴                                | 1.01 × 10⁻⁴                      | 3.51 × 10⁻³       | 7               | 0.99
| CN         | 3–663 × 10⁻⁴                                | 1.32 × 10⁻²                      | 2.62 × 10⁻²       | 11              | 0.95
| MX         | 4–34494 × 10⁻⁴                              | 2.56 × 10⁻⁷                      | 3.57 × 10⁻⁶       | 1               | 0.99
| CH         | 4–1131 × 10⁻⁴                               | 2.26 × 10⁻²                      | 6.87 × 10⁻²       | 13              | 0.94

*Lower concentration at which a chemical induced significant cytotoxicity as compared to the negative control determined by ANOVA test.
Concentration at which cell density was reduced by 50% as compared to the negative control determined by regression analysis.
Coefficient of determination for curve-fitting analysis.*
To meet the new regulation promulgated by the US Environmental Protection Agency (EPA), many drinking water utilities have changed from chlorine to chloramine disinfection. However, this change may increase nitrogenous DBPs, such as HANs, which are unregulated DBPs \((30, 31)\). A study reported that the cytotoxicity or genotoxicity of the HANs may be related to two potential electrophilic reactive centres: (i) displacement of a halogen atom at the α carbon by \(S_N^2\) reaction and (ii) addition at the partially positively charged carbon of the cyano group \((21)\). The \(S_N^2\) reactivity of alkyl bromide is 50 times greater than that of alkyl chloride. The potential of nucleophilic addition of the HANs is dependent on the degree of halogenations \((22)\). Our data showed that DBN and BCN were among the most potent HANs tested in both assays, whereas DCN and CN were the least potent. The higher toxic potency of bromo-HANs relative to chloro-HANs agreed with the \(S_N^2\) structure–activity relationship (SAR) expectation. In contrast to HAAs, the toxic potency of HANs increased with increasing number of halogenated atoms. The relative order of TCN > DCN > CN for cytotoxic and genotoxic potency suggests that nucleophilic addition to the cyano carbon could also contribute to the toxicity.

Although CN is denoted as high-priority DBP by the US EPA, it produced a negative result in the SOS/\(umu\) assay. CN generally tends to be inactive in a number of \textit{in vitro} genotoxicity assays, presumably because of its high cytotoxicity \((32)\). However, the cytotoxicity data of CN presented in this article do not support this hypothesis. CN was the least cytotoxic DBP among the HANs tested.

In previous studies, some DBPs (MX, IA, BA, CA, DCA, DBA and TCA) were analysed for their cytotoxicity in \textit{S. typhimurium} TA100 \((15, 18)\). This permitted a comparison of cytotoxicity of a set of DBPs in different cytotoxicity assays. We found that there is a significant correlation between the two bacterial cytotoxicities \((r = 0.821, P = 0.023)\). Thus, microplate-based cytotoxicity assay established in the present study is suitable for determining the cytotoxicity of DBPs. Moreover, the TA1535/pSK1002 strain is used to simultaneously measure genotoxic and cytotoxic effects, which has the advantage of being easy to handle and less time consuming for

![Figure 2. Genotoxicity of 13 DBPs in \textit{S. typhimurium} TA1535/pSK1002. Genotoxicity assessment was scored as induction ratios (\(i_r\)). DBP concentrations are all presented on a logarithm (base 10) scale. The \(R^2\) for each concentration–response curve ranges from 0.87 to 0.99.](image-url)
preliminary screening of toxicants. However, because induction of the SOS response may result in cell-division arrest and filamentous growth in *S. typhimurium*, cytotoxicity based on a reduction in cell density, can also be a side effect of genotoxicity effects. Taken together, the cytotoxicity assay presented here is recommended to be used in combination with other biotests, such as Microtox® test, which measures cellular metabolic activity, to deliver the most reliable information for toxicity testing. Several studies found that DBP cytotoxicity is significantly correlated with genotoxicity as detected by SCGE and HGPRT in CHO cells (14,33). In the current study, we obtained a similar result ($\tau = 0.946$; $P < 0.001$). This result demonstrates that DBP cytotoxicity is highly related to genotoxic potency in *S. typhimurium*.

Given that many findings confirm that the genetic damage induced by an exogenous compound is closely related to carcinogenesis, genotoxicity and mutagenicity tests have been mainly used to predict carcinogenicity. A study revealed that a genotoxicant detected by the SOS/umu assay can be predicted to be a rodent carcinogen with a high degree of certainty (93%) (12). Among the 13 tested DBPs, DCA induced hepatic tumours in mice and rats (2). While a number of studies suggest some potential mechanisms may be responsible for DCA-induced carcinomas in animals, none of them provide a satisfactory mode of action for the carcinogenicity of DCA. Whether genotoxic effects of DCA account for the carcinogenic response also remains controversial (34). However, recent studies showed DCA produced mutagenic and genotoxic activities in CHO/HGPRT and HepG2/SCGE assays, respectively (23,33). DCA also induced a significant change in DNA damage in the SOS/umu assay in this study. These observations support the idea that genotoxic effects are likely to be involved in the mechanism of DCA carcinogenesis. Besides DCA, three other DBPs, MX, DBA and TCA, which are positive in the SOS/umu assay, have been concluded to be carcinogens in laboratory animals. To date, no carcinogenicity studies are available for BA, IA, DBN, BCN and TCN. However, the observed high genotoxicity of the five DBPs to the *umu* gene warrants evaluation of their carcinogenicity for animal and human safety.

To select the high-priority chemicals from a large number of DBPs found in drinking water, SAR analysis was performed to prioritise these DBPs in accordance with the US EPA (35). Our results suggest that the developed SOS/umu assay is suitable for monitoring the direct genotoxicity of DBPs, particularly for comparing the toxic intensities quantitatively. As necessary, this developed assay could also be used as an important supplement to SAR analysis in prioritising DBPs.

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Conflict of interest statement: None declared.

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