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Analysis of in vivo and in vitro DNA strand breaks from trihalomethane exposure

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Abstract

Background: Epidemiological studies have linked the consumption of chlorinated surface waters to an increased risk of two major causes of human mortality, colorectal and bladder cancer. Trihalomethanes (THMs) are by-products formed when chlorine is used to disinfect drinking water. The purpose of this study was to examine the ability of the THMs, trichloromethane (TCM), bromodichloromethane (BDCM), dibromochloromethane (DBCM), and tribromomethane (TBM), to induce DNA strand breaks (SB) in (1) CCRF-CEM human lymphoblastic leukemia cells, (2) primary rat hepatocytes (PRH) exposed in vitro, and (3) rats exposed by gavage or drinking water.

Methods: DNA SB were measured by the DNA alkaline unwinding assay (DAUA). CCRF-CEM cells were exposed to individual THMs for 2 hr. Half of the cells were immediately analyzed for DNA SB and half were transferred into fresh culture medium and incubated for an additional 22 hr before testing for DNA SB. PRH were exposed to individual THMs for 4 hr then assayed for DNA SB. F344/N rats were exposed to individual THMs for 4 hr, 2 weeks, and to BDCM for 5 wk then tested for DNA SB.

Results: CCRF-CEM cells exposed to 5- or 10-mM brominated THMs for 2 hr produced DNA SB. The order of activity was TBM>DBCM>BDCM; TCM was inactive. Following a 22-hr recovery period, all groups had fewer SB except 10-mM DBCM and 1-mM TBM. CCRF-CEM cells were found to be positive for the *GSTT1-1* gene, however no activity was detected. No DNA SB, unassociated with cytotoxicity, were observed in PRH or F344/N rats exposed to individual THMs.

Conclusion: CCRF-CEM cells exposed to the brominated THMs at 5 or 10 mM for 2 hr showed a significant increase in DNA SB when compared to control cells. Additionally, CCRF-CEM cells exposed to DBCM and TBM appeared to have compromised DNA repair capacity as demonstrated by an increased amount of DNA SB at 22 hr following exposure. CCRF-CEM cells were found to be positive for the *GSTT1-1* gene, however no activity was detected. No DNA SB were observed in PRH or F344/N rats exposed to individual THMs.

Background

Trihalomethanes (THMs) are by-products formed when chlorine is used to disinfect drinking water [1,2]. Trichloromethane (TCM) is most often the predominant THM formed during chlorine disinfection; however, the brominated methanes, bromodichloromethane (BDCM), dibromochloromethane (DBCM), and tribromomethane (TBM) may be formed at levels comparable to or exceeding that of chloroform depending on the concentration of bromine in the water [3]. Total THM concentrations in finished drinking water typically range from 10–100 μg/L in the United States [3]. Epidemiological studies have linked the consumption of chlorinated surface waters to an increased risk of two major causes of human mortality in the United States, colorectal and bladder cancer [4,5].

Recent studies have suggested that exposure to brominated trihalomethanes poses a greater human health risk than exposure to chloroform. Studies in *Salmonella* have shown that brominated THMs are metabolized by GSTT1-1 into mutagenic compounds [6,7]. Additionally, F344/N rats exposed to brominated THMs in drinking water developed colonic aberrant crypt foci (ACF) [8,9]. ACF are considered to be early putative preneoplastic lesions of colon neoplasia [10,11].

Chloroform has been uniformly negative in genetic toxicity assays, whereas the data for the genotoxicity of the brominated THMs have been mixed [12]. The purpose of this study was to examine the ability of the THMs to induce DNA strand breaks (SB) in (1) CCRF-CEM human lymphoblastic leukemia cells, (2) rat hepatocytes exposed in vitro, and (3) rats exposed by gavage or drinking water. DNA SB were measured by the DNA alkaline unwinding assay (DAUA).

Materials and methods

Chemicals

Bromodichloromethane (BDCM; 98+% stabilized with potassium carbonate; CAS 75-27-4), dibromochloromethane (DBCM; 98+%; CAS 124-48-1), tribromomethane (TBM; 99+%; CAS 75-25-2), methyl methanesulfonate (MMS; CAS 66-27-3), and dimethylnitrosamine (DMNA; CAS 62-75-9) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Trichloromethane (TCM; 99+%; CAS 67-66-3) was obtained from Fisher Scientific Co. (Cincinnati, OH). Sodium lauryl sarcosinate (SLS), dithiothreitol (DTT), ethylenediamine-tetracetic acid (EDTA), collagenase Type IV and dexamethasone were from Sigma Chemical Co. (St. Louis, MO). Williams Medium E (WME), Hepes buffer (1 M), fetal bovine serum (FBS), gentamicin and all other cell culture products were purchased from GIBCO (Gand Island, NY). Hydroxylapatite gel (HTP-DNA grade) was obtained from BioRad Laboratories (Richmond, CA). Emulphor (a polyoxyethylated vegatable oil) was from GAF Corp. (Linden, NJ). DNA-specific Hoechst dye 33258 (Bisbenzimide) was purchased from Calbiochem Behring Corp. (La Jolla, CA).

CCRF-CEM cell culture

The CCRF-CEM human lymphoblastic leukemia cell line (American Type Culture Collection, Rockville, MD) was grown in RPMI 1640 medium supplemented with 10% FBS, 25 mM Hepes, 20-mM L-glutamine, and 50-μg/mL gentamicin. The cells were suspended in culture medium without serum at a concentration of 2×10^6 cells/mL, and the test chemicals were added into 2 mL of this suspension. After a 2-hr exposure, the cells were centrifuged and resuspended in 1 mL of ice cold PBS/EDTA and immediately subjected to the DNA SB assay. Viability of the cells following treatment was determined by trypan blue exclusion. In order to study the ability of the cells to recover after removal of the test chemical, half of the cells following the 2-hr exposure were centrifuged and resuspended in fresh culture medium without the test chemical and incubated for an additional 22 hr. The preparation of cells for the DNA SB assay was the same as described for the 2-hr treatment. CCRF-CEM cells are essentially devoid of cytochrome P450 enzyme activity [13].

Rat hepatocyte isolation and culture

Rat hepatocytes were prepared by a two-step liver perfusion method [14] as modified by [15]. Isolated hepatocytes were suspended in Williams medium E (WME) containing 10% FBS, 25-mM hepes, 2-mM L-glutamine, 30-nM dexamethasone, 0.5-μM insulin and 50-μg/mL gentamicin. The cells were incubated for 2 hr at 37EC for attachment, and the test chemicals were added in serum-free medium. After 4-hr of treatment, the medium was removed and tested for lactate dehydrogenase activity (LDH; Sigma Kit No. 228) as an index of cytotoxicity. The cell cultures were rinsed and scraped into 3 mL of ice cold modified Seligmann Balanced Salt Solution (SBSS: 0.13-M NaCl, 2.68-mM KCl, 17.8-mM sodium acetate, 0.36-mM NaH_2PO_4, 0.73-mM KH_2PO_4, 5.55-mM D-glucose, 0.14-mM $NaHCO_3$, 17-mM ascorbic acid, pH 7.2) freshly supplemented with 10-mM DTT and 0.1% nonidet P-40 and were gently homogenized with a dounce homogenizer. The cell pellets were then collected via centrifugation and resuspended in 1-mL PBS/EDTA for the DAUA assay.

Animals and treatments

Male F344/N rats (250–300 g) were purchased from Charles River (Wilmington, MA) and maintained on Purina Laboratory Chow *ad libitum*. Rat exposures were conducted in three stages: a single oral gavage of each THM followed by sacrifice after 4 hr, a 2-week exposure of each THM in drinking water and a 5-week exposure to BDCM in drinking water. In the 4-hr study, test animals

received a single oral gavage dose of test compound (0.3 or 0.6 mM/kg) in deionized water or deionized water containing 0.25% emulphor and sacrificed after 4 hr. Control animals received the appropriate vehicle only. DMNA (2 mmol/kg) was used as the positive control compound. In the 2-week study, rats were exposed to the test chemicals (0.6, 1.2 or 2.4 g/L) in drinking water except TBM, which was given in deionized water containing 0.25% emulphor because of its low solubility in water. The control and solvent control animals were given deionized water and deionized water containing 0.25% emulphor, respectively. In the 5-week study, rats were exposed to BDCM (0.6, 1.2 or 2.4 g/L) in drinking water. Body weights and water consumption were recorded for each exposure group. All aspects of the studies were conducted in facilities certified by the American Association for Accreditation of Laboratory Animal Care in compliance with the guidelines of that association and the NERL Animal Care Committee.

Isolation of liver, kidneys and duodenum cells from rats

Treated rats were sacrificed and 1.5 g of the superior-anterior lobe of the liver was used for cell isolation. The procedure of liver cell isolation from rats for DAUA assay were as follows. The livers were first pressed through a cold stainless steel tissue press, mixed gently with 12-mL SBSS and then filtered through an 8-layer cotton gauze pad. A 1-mL aliquot of liver cells was centrifuged at 100 × *g* and resuspended in 1-mL ice cold PBS/EDTA for the DAUA assay. The isolation of kidney cells was performed in a similar manner. The preparation of duodenum epithelial cells were as follows. A 5-cm segment of duodenum was cut off and flushed with 5-mL of pH 9 buffer (0.114-M Tris, 0.077-M NaCl, pH 9) at 0°C. The segments were cut open, and the intestinal mucus membrane was scraped off using a spatula. The cells were mixed with 5-mL of buffer (pH 9) and drawn through a 12-gauge needle five times. For the DAUA, 1-mL of this cell suspension was used.

DNA Alkaline Unwinding Assay (DAUA)

The DNA alkaline unwinding assay estimates the extent of primary DNA damage based upon the fraction of single- (ss) and double- (ds) stranded DNA. The following types of DNA damage; alkali labile sites, adducts, oxidative damage, dimers, depurinisation, depyrimidation, and desamination have been shown to increase the amounts of ss-DNA following alkaline denaturing (unwinding). The fraction of ds-DNA remaining after alkaline treatment is calculated by dividing the amount of ds-DNA by the total (ss- plus ds-) DNA. This gives a fraction of DNA remaining as ds-DNA and is used as an estimate of DNA damage.

The DAUA was performed according to [16]. Briefly, the treated cells were lysed in 1 mL of 0.1-N NaOH. The time for the unwinding was 1 hr for CCRF-CEM cells and 45 min for primary rat hepatocytes, liver, kidney and intestinal cells. Solutions were neutralized with 1 mL of 0.1-N HCl and 0.25-mL of 2% SLS/20 mM EDTA followed by 5 sec of sonication. Single- (ss) and double- (ds) stranded DNA were separated on a hydroxylapatite column at 60°C. Hoechst Dye 33258 was added at a concentration of 4×10^{-7} M, and the amount of DNA in each fraction was determined fluorimetrically using a Shimadzu RF-5000 U spectrofluorometer set at an excitation wavelength of 350 nm and an emission wavelength 465 nm. The fraction (F) of ds-DNA remaining after alkaline unwinding was calculated by dividing the amount of ds-DNA by the total (ss- plus ds-) DNA.

The relationship between the F-value and the number of DNA SB induced per cell (Ni) as a function of concentration or the test chemical has been determined previously for CCRF-CEM cells [16,17]. The number of DNA SB induced (Ni) per cell was calculated as $Ni = -6.1 \times 10^4 [\ln (F_T/F_O)]$, where Ni is the number of DNA SB per cell induced, F_T and F_O are the F values for the treated cells and control cells, respectively. The decrease in the natural logarithm of the F-value is inversely proportional to the increase in the number of induced DNA SB. Due to the quantitative and qualitative differences in the activity of the DNA repair enzymes between cell and tissue types, this equation should not be used to estimate (based on F-value) the absolute number of DNA SB in other cell lines. Nevertheless, the decreasing F-value indicates increasing DNA SB in all cell types.

Multiplex PCR characterization of GSTT1-1

DNA was extracted from CCRF-CEM cells by using the standard proteinase K, phenol/chloroform technique and reconstituted in Tris-EDTA buffer (pH 8.0). DNA (100 – 400 ng titrations) was genotyped by multiplex polymerase chain reaction (PCR) [18] with minor modifications. *GSTT1-1* was co-amplified with *GSTM1* and beta globin as an internal positive control. External controls included samples from a subject with known positive genotype for *GSTT1-1* and *GSTM1* and samples from a subject with known null genotype for both enzymes. The Qiagen HotStar Taq DNA Polymerase method was used to enhance PCR and avoid cross-contamination from external hot-starting. A final concentration of 6.2-mM magnesium chloride was used in the reaction (total volume = 35 μl). Five μL of PCR product was mixed with 2 μL load dye (bromophenol blue, xylene cyanole, ficoll) and electrophoresed alongside a 50-base-pair ladder marker (Invitrogen, cat. no. 10416-014) in 1X Tris-Borate-EDTA pH 8.0 buffer on 1.5 % agarose, 0.5 μg/mL ethidium bromide gel at 82 mA for 40 min. Gel was digitally photographed on an ultra-violet lightbox using a Kodak Digital Camera System.

Table 1: Results of DNA damage (measured as strand breaks by the DNA alkaline unwinding assay, DAUA) from CCRF-CEM cells exposed to methyl methanesulfonate (MMS), trichloromethane (TCM), bromodichloromethane (BDCM), dibromochloromethane (DBCM), and tribromomethane (TBM) for 2 hr.

Treatments	Break/cell/μM (2 hr)	%Damage (2 hr)	Via	Break/cell/μM (24 hr)	%Damage (24 hr)	Via	%Recovery
Control	---	0 ± 0.54	94	---	0 ± 1.29	96	---
MMS-0.5 mM	50020 ± 2810*	56.01 ± 2.47*	89	12740 ± 450*	18.84 ± 2.87*	75	74.53
TCM-1 mM	1830 ± 43	3.02 ± 2.29	94	220 ± 3	0.39 ± 1.35	96	87.98
5 mM	3180 ± 33	5.13 ± 1.02	95	470 ± 11	0.82 ± 2.31	94	85.22
10 mM	1530 ± 17	2.51 ± 1.11	88	490 ± 4	0.82 ± 0.74	94	67.97
BDCM-1 mM	2010 ± 45	3.31 ± 2.14	92	0	-1.09 ± 2.07	96	100
5 mM	9290 ± 525*	14.17 ± 4.85*	92	0	-1.24 ± 1.52	92	100
10 mM	8590 ± 205*	13.19 ± 2.09*	87	2540 ± 31	4.16 ± 1.16	89	70.43
DBCM-1 mM	3220 ± 140	5.16 ± 4.11	91	0	-2.45 ± 0.70	92	100
5 mM	7440 ± 112*	11.55 ± 1.34*	86	4430 ± 128	7.03 ± 2.68	86	40.46
10 mM	14990 ± 870*	21.83 ± 4.53*	85	16920 ± 937*	24.24 ± 4.18*	76	-12.86
TBM-1 mM	3150 ± 43	5.13 ± 1.29	91	4280 ± 95	6.80 ± 2.07	85	-35.87
5 mM	16800 ± 22*	24.12 ± 0.10*	90	30490 ± 2110*†	39.36 ± 4.23*	45	-81.49
10 mM	34030 ± 1212*	42.79 ± 2.04*	78	53540 ± 6829*†	58.43 ± 5.30*	12	-57.33

*Significantly different when compared to control ($p < 0.05$) †These data are not considered significant due to associated high cell mortality that may be the result of secondary DNA damage from cytotoxic injury. Following exposure, half of the cells were immediately subjected to the DAUA, and the remaining half were suspended in fresh medium for 22 hr, then subjected to DAUA to access recovery. DNA damage is reported as breaks per cell for a given concentration and percent damage compared to control, ± standard error. Viability (Via) was performed by the trypan blue exclusion assay and is reported as the percent of living cells compared to control. The viability was 98% at the time of treatment. Recovery is reported as the percent difference between the 2 and 24 hr DNA damage count. Each treatment was run in triplicate.

GSTT1-1 enzyme activity assay

Fresh human CCRF-CEM cells were harvested by centrifugation (3,000 × *g*, 10 min) and the cells washed with cold 0.9% w/v saline. The washed cells were then resuspended in 0.5 mL of ice-cold 10 mM potassium phosphate (pH 6.5) and sonicated on ice (4 pulses, 30 s per pulse). The resulting homogenate was centrifuged at 20,000 × *g* (4°C, 30 min). The soluble fraction was filtered (0.22 μ) and assayed for protein content by the method of Bradford. The protein concentration of the extract was 7.37 mg/mL and the GSTT1-1 activity toward the substrate 1,2-epoxy-3-(4'-nitrophenoxy)propane (ENPP) was determined in duplicate by a spectrophotometric assay described previously, using $\Delta\varepsilon = 0.5\ mM^{-1}\ cm^{-1}$ [19]. B6C3F1 mouse liver cytosol was used as a positive control for this assay (specific activity, 202.5 nmol/min/mg protein, $n = 2$).

Statistical analysis

Data were tested for normality, and a oneway analysis of variance was performed on the group data. Analysis to determine significant increases from corresponding treatment controls was performed by a Dunnett's T-test at $p < 0.05$. Analysis was performed using SPSS version 11.0 software (SPSS Inc., Chicago, IL).

Results

CCRF-CEM trihalomethane exposure

Bromodichloromethane (BDCM), dibromochloromethane (DBCM), and tribromomethane (TBM) at 5 or 10 mM significantly induced DNA SB in CCRF-CEM cells after a 2-hr exposure (Table 1). Trichloromethane (TCM) was inactive in this study. MMS (0.5 mM), a direct-acting genotoxin used as the positive control chemical, induced significant DNA SB in CCRF-CEM cells. There was no significant decrease in cell viability among all concentrations tested. MMS induced the most DNA SB at 50,020 SB/cell/μM (56.01% damage), whereas TBM was the most potent THM, inducing 16,800 and 34,030 SB/cell/μM (24.12 and 42.79% damage) at 5 and 10 mM, respectively. The numbers given for DNA SB induced per cell were calculated as described by [16,17].

The ability of CCRF-CEM cells to recover following THM exposure was examined by incubating the cells for an additional 22 hr in fresh medium then analyzing for DNA SB. DNA SB recovery was observed in TCM, BDCM at 1 and 5 mM and DBCM at 1 mM. Cell viability was also increased for the prior mentioned THMs. However, 10-mM DBCM and 1-mM TBM treated cells showed increased levels of DNA damage, indicating lack of recovery. Moreover, 5- and 10-mM TBM treated cells also showed no recovery, however, these data are not considered biologically significant due to associated high cell mortality that may be the result of secondary DNA damage from cytotoxic injury. CCRF-CEM cells were positive for the presence of the *GSTT1-1* gene (Fig. 1), however, GSTT1-1 activity was not detected in the soluble

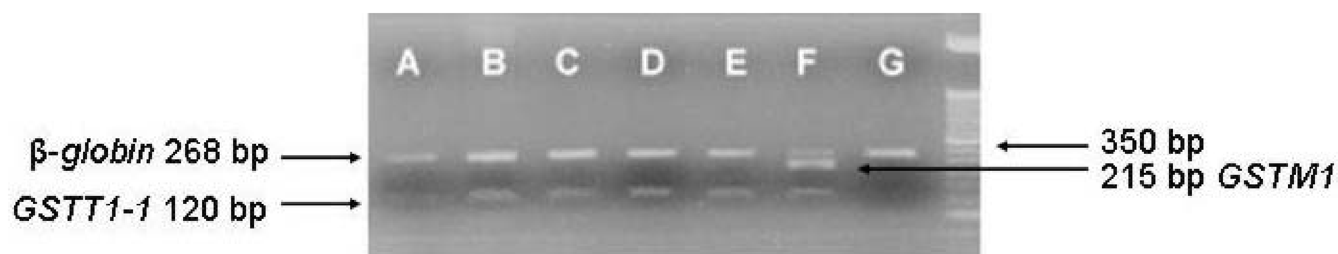

Figure 1
Multiplex PCR characterization of *GSTT1-1* in CCRF-CEM cells. *GSTT1-1* was co-amplified with *GSTM1* and beta globin as an internal positive control. Lanes A through E are CCRF-CEM cells at 50.5, 204, 408, 816, and 74 ng DNA, respectively. Lanes F and G are external controls (320 ng DNA) with lane F showing a subject positive for both *GSTT1-1* and *GSTM1* with lane G showing a subject null for both enzymes. DNA marker is a 50-bp ladder (Invitrogen).

protein fraction by the spectrophotometric assay utilized [19].

Rat hepatocyte trihalomethane exposure
Significant increases in DNA SB that were not associated with cytotoxicity were not observed in primary rat hepatocytes (PRH) exposed to individual THMs for 4-hr. Although significant increases in DNA SB were observed at 10-mM DBCM and 10-mM TBM, this finding correlated with high cell mortality and may be the result of secondary DNA damage from cytotoxic injury (Table 2). Cytotoxicty was assessed by measuring the amount of cellular lactate dehydrogenase (LDH) released at the conclusion of the 4-hr exposure. MMS- (0.5 mM) exposed PRH produced levels of DNA SB similar to those observed in MMS-exposed CCRF-CEM cells. BDCM at 10 mM and DBCM at 5 mM were cytotoxic to PRH, although no evidence of DNA SB were observed. Similar to CCRF-CEM cells, TCM did not produce significant DNA SB or cytotoxicity to PRH.

Table 2: Primary rat hepatocytes (PRH) were exposed to methyl methanesulfonate (MMS-positive control), trichloromethane (TCM), bromodichloromethane (BDCM), dibromochloromethane (DBCM), and tribromomethane (TBM) for 4 hr.

Treatments	% Damage	LDH (% Max)
Control	0 ± 0.42	4 ± 0.18
MMS-0.5 mM	53.11 ± 4.13*	11 ± 0.72*
TCM-1 mM	0	4 ± 0.03
5 mM	0	6 ± 0.09
10 mM	0	5 ± 0.69
BDCM-1 mM	0	7 ± 0.57
5 mM	2.24 ± 4.31	11 ± 0.45
10 mM	3.39 ± 1.88	41 ± 0.87*
DBCM-1 mM	10.0 ± 4.25	7 ± 0.22
5 mM	8.64 ± 6.04	32 ± 3.10*
10 mM	71.58 ± 4.55*†	92 ± 1.27*
TBM-1 mM	0	5 ± 0.25
5 mM	0.76 ± 4.72	14 ± 0.50
10 mM	65.90 ± 4.66*†	89 ± 3.75*

*Significantly different when compared to control ($p < 0.05$) †These data are not considered significant due to associated high cell mortality that may be the result of secondary DNA damage from cytotoxic injury. DNA damage (measured as strand breaks by the DNA alkaline unwinding assay, DAUA) is reported as percent control damage ± standard error. The cytotoxicty of PRH was measured by the amount of cellular lactate dehydrogenase (LDH) at the conclusion of the exposure. Mean values are the percent maximum release ± SE; DAUA and LDH assays were performed in triplicate.

Rat trihalomethane exposure
F344/N rats administered a single oral gavage dose of each THM (0.3 or 0.6 mM/kg) in 0.5% emulphor and analyzed 4 hr later showed no DNA SB in liver, kidney, or duodenum epithelial cells when compared to control animals. Furthermore, in the 2-week study, only the positive control, DMNA (2 mM/kg), produced significant DNA SB in rat liver and kidney epithelial cells but not in duodenum epithelial cells. Rats exposed to BDCM (0.6, 1.2 or 2.4 g/L) in drinking water for 5 weeks showed no induction of appreciable DNA SB in liver, kidney, or duodenum epithelial cells (data not shown).

Discussion
CCRF-CEM cells exposed to the brominated THMs at 5 or 10 mM for 2 hr showed a significant increase in DNA SB when compared to control cells. TCM was inactive in this study. At these concentrations, cell viability ranged from 78–94%. Following the 2-hr exposure, half of the exposed cells were transferred to clean media for 22 hr then tested for DNA SB. BDCM at 1 and 5 mM and DBCM at 1 mM showed 100% DNA SB recovery; however, DBCM at 10 mM and TBM at 1 mM showed additional DNA SB. TBM at 5- and 10-mM also showed additional DNA SB, however we do not believe this to be biologically significant

due to the high degree of cell mortality that may have elevated the levels of DNA SB via secondary damage from cytotoxic injury. All treatments that showed positive recovery also showed acceptable and near identical viability at 2 and 24 hr. These data imply that DBCM at 10 mM and TBM at 1 mM continue to damage DNA following removal of cells from exposure media, and TBM at 5- and 10-mM also continue to damage DNA concurrently with additional cell mortality over 22 hr.

A possible explanation for the lack of recovery in DBCM and TBM exposed CCRF-CEM cells may be the higher octanol/water partition coefficients (log P_{ow}) of the more brominated THMs. The P_{ow} is a measure of a compound's lipophilicity and an indication of its ability to cross cell membranes. All of the THMs are slightly lipophilic with a P_{ow} of 1.97, 2.1, 2.24, and 2.38 for TCM, BDCM, DBCM, and TBM, respectively [20,21]. Furthermore, the partition coefficients of TCM and BDCM are 203 and 526 in fat, respectively and 21.1 and 30.6 in liver respectively [22,23]. Although the CCRF-CEM cells that were examined for DNA SB recovery were placed into fresh media following the 2-hr exposure, the more lipophilic THMs may have remained in the cells longer and thus contributed to additional DNA SB.

Toxicological studies have demonstrated that 50 and 100 mg/kg BDCM and 100 and 200 mg/kg TBM administered by corn oil gavage induced colorectal adenocarcinoma in male and female F344/N rats [24,25]. The increased incidence of colorectal cancer was concordant with findings from epidemiologic studies. However, a recently completed 2-year bioassay of BDCM administered in the drinking water to male F344/N rats failed to demonstrate colorectal neoplasia [26]. Although BDCM in the drinking water did not increase the incidence of colorectal cancer in rats, exposure to the brominated THMs in the drinking water did induce aberrant crypt foci (ACF) [8]. ACF are collections of abnormal colonic crypts that are characterized by hyperproliferation, increased size, expanded pericryptal zones, and elongated or serrated crypt lumens. ACF are considered to be early putative preneoplastic lesions of colon neoplasia [10,11].

Studies have shown that alterations in the cells ability to repair DNA is an early event in the formation of ACF [27,28]. In this study, CCRF-CEM cells exposed to DBCM and TBM appeared to have compromised DNA repair capacity as demonstrated by an increase accumulation of DNA SB following exposure. Current work in our lab on F344/N rats show a significant increase in ACF as the number of bromine atoms on the THM increases. One possible explanation may be that rats exposed to brominated THMs may have decreased DNA repair capacity and, as a result, be more disposed to ACF development. Future studies are planned to examine this hypothesis.

The relationship between brominated THM metabolism and toxicity is not completely understood; however, a theta-class glutathione S-transferase known as GSTT1-1, the protein product of the *GSTT1-1* gene, catalyzes the formation of mutagenic intermediates from brominated THMs. [29-31]. These intermediates have recently been shown to covalently bind DNA and produce deoxyguanosine adducts in vitro [32]. *GSTT1-1* is polymorphic in humans with 20–25% of Caucasians and 50% of Asians having a homozygous deletion of this gene resulting in a null genotype [33-35]. GSTT1-1 has also been detected in F344/N rat tissues [36,37]. In vitro results suggest that people with at least one copy of the *GSTT1-1* gene might be more susceptible to the genotoxic effects of the brominated THMs than those missing the gene.

In this study, the presence of the *GSTT1-1* gene was confirmed in CCRF-CEM cells, however no enzyme activity was detected. This implies that CCRF-CEM human lymphoblastic leukemia cells do not express the GSTT1-1 enzyme or that levels of functional enzyme were less than the detection limit of the assay. This result correlates with the finding that human lymphocytes also lack GSTT1-1 activity [38,39]. Nevertheless, it has been reported that CCRF-CEM cells do express GST activity toward the universal GST substrate 1-chloro-2,4-dinitrobenzene [40], suggesting the expression of GST alpha, mu, and pi isoforms. These findings raise the question of whether brominated THMs act by two different mechanisms to: 1) induce GSTT1-1-dependent mutations in *Salmonella*, and 2) produce DNA SB in CCRF-CEM cells, apparently independent of significant metabolism by GSTT1-1.

PRH exposed to 10-mM DBCM or TBM for 4 hr produced significant DNA SB. These data are similar to those reported for CCRF-CEM cells where both the high 10-mM concentrations of DBCM and TBM caused significant increases in DNA SB. A 2-fold increase in DNA SB was observed between CCRF-CEM cells exposed to 5- or 10-mM DBCM or TBM; however, an 8- and 86-fold increase, respectively was seen in PRH. At low concentrations, PRH may have utilized protective or repair mechanisms that were less efficient in the CCRF-CEM cells, which are not known to express any cytochrome P450 enzyme activity and are essentially devoid of enzymes that can activate carcinogens to DNA-reactive metabolites [13]. A comparison of DNA SB dose-response linearity between CCRF-CEM cells and PRH for DBCM and TBM yielded r^2 values of 0.990 and 0.991 for CCRF-CEM cells, respectively, and r^2 values of 0.576 and 0.622 for PRH. This may suggest a threshold concentration in PRH for DNA damage between 5 and 10 mM for both DBCM and TBM. Further-

more, cytotoxicity was increased proportionally with dose and may have contributed to the levels of DNA SB observed at 10-mM DBCM or TBM.

No DNA SB in liver, kidney, or duodenum epithelial cells were observed in F344/N rats exposed to each THM by a single oral gavage (0.3 or 0.6 mM/kg), for 2 weeks in drinking water (0.6, 1.2 or 2.4 g/L), or BDCM for 5 weeks in drinking water (0.6, 1.2 or 2.4 g/L). Although no DNA SB were observed in the tissues listed above, future studies are planned to evaluated DNA damage and repair in the colon of F344/N rats following THM exposure.

Conclusions

CCRF-CEM cells, exposed to the brominated THMs at 5 or 10 mM for 2 hr showed a significant increase in DNA SB when compared to control cells. Additionally, CCRF-CEM cells exposed to DBCM and TBM appeared to have compromised DNA repair capacity as demonstrated by an increased amount of DNA SB at 22 hr following exposure. CCRF-CEM cells were found to be positive for the *GSTT1-1* gene, however no activity was detected. PRH exposed to 10-mM DBCM or TBM for 4 hr produced significant DNA SB, however, cytotoxicity was increased proportionally with dose and may have contributed to the observed increase. No DNA SB were observed in liver, kidney, or duodenum epithelial cells of F344/N rats exposed to individual THMs by a single oral gavage, for 2 weeks in drinking water, or BDCM for 5 weeks in drinking water.

Authors' contributions

Author DG performed data and statistical analysis, and wrote the manuscript. Authors AD and LC conceived of the study and LC conducted the CCRF-CEM, rat hepatocyte and in vivo testing. NH examined CCRF-CEM cells for the presence of *GSTT1-1* and authors MR and RP performed the GSTT1-1 activity assay and aided in the discussion. All authors read and approved the final manuscript.

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