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Cytotoxicity, genotoxicity, and mutagenicity of 1-chloro-2-hydroxy-3-butene and 1-chloro-3-buten-2-one, two alternative metabolites of 1,3-butadiene

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Abstract

The cytotoxicity, genotoxicity, and mutagenicity of 1-chloro-2-hydroxy-3-butene (CHB), a known in vitro metabolite of the human carcinogen 1,3-butadiene, have not previously been investigated. Because CHB can be bioactivated by alcohol dehydrogenases to yield 1-chloro-3-buten-2-one (CBO), a bifunctional alkylating agent that caused globin-chain cross-links in erythrocytes, in the present study we investigated the cytotoxic and genotoxic potential of CHB and CBO in human normal hepatocyte L02 cells using the MTT assay, the relative cloning efficiency assay and the comet assay. We also investigated the mutagenic potential of these compounds with the Ames test using *Salmonella* strains TA1535 and TA1537. The results provide clear evidence for CHB and CBO being both cytotoxic and genotoxic with CBO being approximately 100-fold more potent than CHB. Interestingly, CHB generated both single-strand breaks and alkali-labile sites on DNA, whereas CBO produced only alkali-labile sites. CHB did not directly result in DNA breaks, whereas CBO was capable of directly generating breaks on DNA. Interestingly, both compounds did not induce DNA cross-links as examined by the comet assay. The Ames test results showed that CHB induced point mutation but not frameshift mutation, whereas the toxic effects of CBO made it difficult to reliably assess the mutagenic potential of CBO in the two strains. Collectively, the results suggest that CHB and CBO may play a role in the mutagenicity and carcinogenicity of 1,3-butadiene.

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Conflict of interest

The authors declare no conflict of interest.

Keywords

1; 3-butadiene; 1-chloro-2-hydroxy-3-butene; 1-chloro-3-buten-2-one; genotoxicity; mutagenicity; comet assay

Introduction

1,3-Butadiene (BD) is a petrochemical manufactured in high volume to be used as an intermediate in the production of polymers, elastomers, and other chemicals. It is also an air pollutant and has been classified as a human carcinogen. BD is a multisite carcinogen in laboratory rodents and is associated with leukemia and lymphohematopoietic cancers in humans (Melnick and Sills, 2001; IARC, 2008; Owen and Glaister, 1990; NTP, 1993; EPA, 2002; Kirman et al., 2010). The presence of BD in the urban air is ubiquitous because automobile exhaust is the principal source, accounting for 78.8% of the total emission amount (EPA, 2002). In addition, BD is also present in cigarette smoke and in the exhaust of incomplete combustion of biomass (EPA, 2002; Eschner et al., 2011).

BD is not a direct-acting carcinogen. Rather, its mutagenicity/carcinogenicity requires metabolic activation. The first step in the bioactivation of BD is the cytochrome P450-mediated biotransformation into 3,4-epoxy-1-butene (EB) (Malvoisin and Roberfroid, 1982; Elfarra et al., 1991), which can be further converted to 1,2,3,4-diepoxybutane (DEB) and 3,4-epoxy-1,2-butanediol (EBD) (Malvoisin and Roberfroid, 1982; Seaton et al., 1995; Boogaard and Bond, 1996; Krause and Elfarra, 1997). EB, DEB, and EBD are all direct-acting mutagens/carcinogens (Cochrane and Skopek, 1994a; Cochrane and Skopek, 1994b). These compounds contain oxirane moieties, which are reactive towards DNA and proteins. Many DNA/protein adducts of these metabolites have been reported (Albertini et al., 2010). Their mutagenicity and genotoxicity have been extensively investigated as well, although most studies have been focused on DEB (Albertini et al., 2010).

An alternative metabolic pathway is a myeloperoxidase-mediated oxidation of BD in the presence of hydrogen peroxide and chloride ion, which leads to the formation of EB and 1-chloro-2-hydroxy-3-butene (CHB) (Duescher and Elfarra, 1992). When the concentration of chloride ion is higher than 50 mM, CHB is the major product. The metabolic pathway is expected to be active in immune and bone marrow cells, e.g., neutrophil granulocytes. Neutrophil granulocytes contain large amounts of myeloperoxidase and high concentrations of chloride ion (80–111 mM) (Nauseef, 2007), and can generate high concentrations of hydrogen peroxide (which can reach millimolar level) when the cells are stimulated by invading microorganisms or upon oxidative burst as part of the inflammatory response.

Like EB, CHB can also undergo further biotransformation into other compounds. Recently, we demonstrated that CHB could be oxidized to 1-chloro-3-buten-2-one (CBO) by alcohol dehydrogenase and rat liver cytosol (Elfarra and Zhang, 2012). CHB may also be converted to CBO by other enzymes, such as cytochrome P450 2E1 and 3A4, since 3-butene-1,2-diol, a structural analog of CHB, can be oxidized to hydroxymethyl vinyl ketone by cytochrome P450s (Krause et al., 2001). CBO is a Michael acceptor with bifunctional alkylating ability (Elfarra and Zhang, 2012). In reactions with glutathione (GSH) it readily formed a CBO-di-GSH conjugate. Globin-chain cross-links were also detected upon in vitro incubation of CBO with freshly-isolated erythrocytes (Elfarra and Zhang, 2012).

Based on their chemical structures, CHB and CBO are expected to be reactive towards other cellular proteins and various biomolecules, such as DNA, suggesting that these chemicals are likely to be genotoxic and mutagenic. However, the cytotoxicity, genotoxicity and mutagenicity of the two compounds have not been examined previously. In the present

study, we investigated the cytotoxicity and genotoxicity of CHB and CBO in human normal hepatocyte L02 cells using the MTT assay, the relative cloning efficiency assay and the comet assay. We also investigated the mutagenic potential of CHB and CBO using the Ames *Salmonella* mutagenicity assay (Ames test) with strains TA1535 and TA1537.

Materials and methods

Reagents

BD (99%) was obtained from Dalian Da'te Gas Ltd. (Dalian, China). Calcium hypochlorite was purchased from Alfa Aesar (Ward Hill, MA, US). Chromium oxide was obtained from Adamas Reagent Company (Shanghai, China). Sodium azide, 9-aminoanthracene, EB, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), Triton X-100, agarose, methyl methanesulfonate (MMS), and GSH were purchased from Sigma-Aldrich (St. Louis, MO, US). Dulbecco's Modified Eagle's Medium and fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY, US). Other reagents were obtained from Sinopharm Chemical Reagent Company (Shanghai, China) and were of analytical reagent grade. CHB was synthesized from BD and calcium hypochlorite as described in the literature (Kadesch, 1946). Its purity was ≥ 98% as determined by GC-MS. CBO was synthesized as reported in our preceding paper (Elfarra and Zhang, 2012). The CBO used in the experiments was purified on a silica gel column and the purity was at least 98% as determined by GC-MS. The pH 7.4 phosphate buffer (100 mM) contained 100 mM KCl.

Cell line and bacteria strains

The immortalized human normal hepatocyte cell line L02 was the gift of Professor Ping-Kun Zhou (Beijing Institute of Radiation Medicine, Beijing, China). It has been used in many studies to evaluate the biological effects of xenobiotics and also investigate the cellular signaling pathways (see references cited in (Wen et al., 2011)). Because we previously used this cell line to investigate the genotoxicity of DEB, EB, and EBD (Wen et al., 2011; Zhang et al., 2012), use of this cell line in the present study would make it possible to compare the results among the used BD metabolites.

Hepatocyte L02 cells were cultured in Dulbecco's Modified Eagle's Media supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37 °C in 5% CO_2. Cells in ~80% confluence were collected by trypsinization and subjected to the comet assay as described below. *Salmonella* strains TA1535 and TA1537 were obtained from American Tissue Culture Collection (Manasses, VA, US). The two strains were selected to examine point mutation and frameshift mutation, respectively (Mortelmans and Zeiger, 2000).

MTT assay

MTT assay was employed to examine the cytotoxicity of CHB and CBO. The CHB concentrations used were 10, 50, 200, and 1000 μM. For CBO, the concentrations initially tested were also 10, 50, 200, and 1000 μM. However, the highest CBO concentration was reduced to 100 μM due to excessive toxicity observed at high concentrations and too strong DNA damage caused by CBO at > 10 μM in the standard comet assay (see below). The final CBO concentrations tested were 10, 20, 30, 40, 50, and 100 μM. L02 cells were incubated with CHB or CBO in FBS-free media at 37 °C for 1 h. The media were discarded and a solution of 10 μl MTT (5 mg/ml) in 90 μl FBS-free medium per well was added. The plates were incubated at 37 °C for 4 h, the media were removed and 100 μl dimethyl sulfoxide was added to each well. The plates were then shaken at ambient temperature for 3 min and the

absorbance at 490 nm was measured. Six independent samples were used at each concentration.

Relative cloning efficiency (RCE) assay

The long-term survivability of cells was examined with the RCE assay. The CHB concentrations selected were 10, 20, 50, 100, 300, 500, and 1000 μM. The highest CBO concentration used was 10 μM because few cells survived at this concentration. The preliminary experiments indicated that CBO at a concentration as low as 0.2 μM showed statistically significant effect, thus two lower concentrations, 0.01 and 0.05 μM, were added. The final concentration series for CBO included 0.01, 0.05, 0.2, 1, 2, 5, and 10 μM. L02 cells (200 per dish) were inoculated in petri dishes. After culture in the incubator overnight, cells were treated with FBS-free fresh media containing CHB or CBO at 37 °C for 1 h. The media were then discarded and cells were cultured for 7–14 days. Colonies formed were fixed, stained with Coomassie and counted. The RCE values (i.e., the survival rates of cells) were determined relative to the corresponding controls. Three independent samples were used at each concentration.

Comet assay

The comet assay (i.e., single-cell gel electrophoresis) is a standard technique to test genotoxicity of chemicals and has been widely used in biomonitoring of human populations, molecular epidemiology, and assessment of DNA damage/repair and oxidative stress (Tice et al., 2000; Collins, 2004; Collins et al., 2008). Multiple variants of comet assay, including the standard comet assay, the pH 11.9 and pH 9 comet assays, and the acellular comet assay, were employed. The standard comet assay detects both single-strand breaks (SSB) and alkali-labile sites (ALS), because in this assay a strongly alkaline electrophoresis solution (pH > 13) is used. At this pH, ALS can be converted into SSB, thus becoming detectable (Miyamae et al., 1997; Tice et al., 2000). To discriminate between ALS and SSB, two variants of comet assay, which are called the pH 11.9 and pH 9 comet assays, were utilized. ALS cannot be converted to SSB at pH ≤ 12.1 (Miyamae et al., 1997), thus being able to be distinguished from SSB (Tice et al., 2000). The discrimination is achieved through comparing the rates of DNA migration observed using the pH 11.9 comet assay with those obtained using the standard comet assay. If a chemical causes equal DNA migration using the two comet assays, the chemical generates only SSB; if it induces DNA migration with the standard comet assay but fails to do so with the pH 11.9 comet assay, the chemical produces only ALS (Tice et al., 2000). However, the pH 11.9 comet assay has an intrinsic drawback that the electrophoresis solution is not very stable (Merk et al., 2000; Wen et al., 2011). As a result, we additionally used the pH 9 comet assay to further confirm the results obtained with the pH 11.9 comet assay (Wen et al., 2011). In addition, the acellular comet assay was utilized to examine if a chemical was capable of directly causing SSB and ALS on DNA (Kasamatsu et al., 1996).

The protocols for these variants were described previously (Wen et al., 2011; Zhang et al., 2012). Briefly, exposure of cells to the chemicals tested was achieved by incubating them in FBS-free media containing the chemicals at 37 °C for 1 h. Cells were subsequently harvested by trypsinization and embedded in agarose gel on glass slides. For the standard comet assay, cells were lysed in the lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10; 1% Triton X-100 and 10% dimethyl sulfoxide were added fresh). DNA was unwound in the electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH > 13) at 4 °C for 40 min and subsequently electrophorised in this solution at 25 V (~300 mA) for 20 min. The pH 11.9 comet assay protocol was the same as the standard one except the electrophoresis conditions. In this protocol, the electrophoresis solution contained 300 mM NaOH and 1 mM EDTA but its pH was adjusted to 11.9 with sulfuric acid and

electrophoresis was performed at 40 V for 30 min (Miyamae et al., 1997; Wen et al., 2011). The pH 9 comet assay protocol was similar to the standard one except that the electrophoresis solution was a Tris-acetate buffer (100 mM Tris, 300 mM sodium acetate, the pH was adjusted to 9.0 using glacial acetic acid) and "unwinding" of DNA was carried out in this buffer at 4 °C for 20 min (Singh and Stephens, 1997; Wen et al., 2011). The acellular comet assay used the same lysis and electrophoresis solutions as the standard comet assay (Kasamatsu et al., 1996), but cells were lysed first, washed (400 mM pH 7 phosphate buffer, 4 °C, 5 min, twice), then exposed to CHB and CBO, washed again, unwound, and electrophorised at 20 V for 10 min (Zhang et al., 2012). Exposure of lysed cells to CHB and CBO was achieved by applying 100 μl of 400 mM pH 7 phosphate buffer containing different concentrations of CHB and CBO to each gel, covering the gels with coverslips and then incubating in a moist chamber at 37 °C for 1 h.

To examine the presence of cross-links, a second genotoxicant, MMS, was employed (Tice et al., 2000). The second genotoxicant generates additional DNA damage and cross-linking can be detected through retardation in the rate of DNA migration. For chemicals that can also generates SSB and ALS, it is important that DNA damage caused by the second genotoxicant is severe enough, otherwise the DNA migration-reducing effect of cross-links might not be detected (Wen et al., 2011). A preliminary experiment was performed to screen an appropriate MMS concentration. It was found that 600 μM MMS caused maximal DNA damage (which reached 70–80%; higher MMS concentration led to such severe damage that DNA images were unable to be observed) and thus was employed to examine the cross-linking effect of CHB and CBO.

For CHB, five concentrations (10, 50, 200, 500, and 1000 μM) were used. For CBO, however, the highest concentration chosen was 10 μM because CBO at higher concentrations (20, 30, 40, and 50 μM) led to excessive DNA damage. As a result, the CBO concentrations finally used in the experiments were 0.5, 1, 2, 5, and 10 μM. Three independent samples were used at each concentration.

Ames test

BD exposure has been associated with both point mutations and frameshift mutations in mammals (Albertini et al., 2010). Thus, two *Salmonella* strains, TA1535 and TA1537, were selected because the DNA sequences in the two strains are designed to detect point mutations and frameshift mutations, respectively (Mortelmans and Zeiger, 2000), and these strains were used previously to characterize BD, EB, and DEB mutagenicity (de Meester et al., 1978; Himmelstein et al., 2001; McCann et al., 1975). All experiments were carried out using the pre-incubation assay procedure as described in the literature (Mortelmans and Zeiger, 2000). Briefly, CHB or CBO dissolved in phosphate buffer was incubated with bacteria at 37 °C for 20 min and then the mixtures were added to the top agar and poured onto plates. Plates were allowed to incubate at 37 °C for 48 h and then revertants were counted. Toxicity was indicated by a decrease in the bacterial lawn or decreases in spontaneous revertant colonies. Each concentration was tested in triplicate. The concentrations used for each compound were chosen from an initial screen to indicate mutagenic potential or toxicity but no chemical would be tested at a concentration higher than 5 mg per plate. For CHB, the following concentrations were used: 0.5, 1, 2, 4, and 5 mg per plate; for CBO, the concentrations were 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, and 2 mg per plate. Sodium azide (2 μg per plate) and 9-aminoanthracene (25 μg per plate) were used as positive controls for TA1535 and TA1537, respectively. EB (5 mg per plate) was also used as a positive control because it is a known mutagenic BD metabolite and gave positive response in the Ames test (Adler et al., 1997; Albertini et al., 2010). A chemical was classified as mutagenic if the mutant frequency was at least three-fold greater than that

of the spontaneous revertant rate. Three independent samples were used at each concentration.

Statistical analyses

The statistical analyses of the results obtained with comet assays were the same as those described in our previous papers (Wen et al., 2011; Zhang et al., 2012). Percentage of DNA in the tail (%Tail DNA) was used as the metric for quantification of DNA migration based on its apparent advantages over other metrics (Hartmann et al., 2003; Collins, 2004; Kumaravel and Jha, 2006; Burlinson et al., 2007; Collins et al., 2008; Lovell and Omori, 2008). One hundred cells per glass slide were selected randomly and the images of comets were analyzed using CASP image-analysis software to obtain the quantitative data of comets (Konca et al., 2003). Each glass slide represented one independent sample. Because the intra-sample data in our experiments did not conform to normal distributions, medians, instead of arithmetic means, were extracted from the data and were subsequently used in the analyses of results on the basis of the recommendation of biostatisticians (Duez et al., 2003; Lovell and Omori, 2008). The values reported in the present study were the means and standard deviations (SD) of at least three independent samples. All experiments were performed at least in triplicate. Student's *t*-test was used to examine the statistical significances of the differences between two values. The p-value for statistical significance was set at 0.05.

Results

The cytotoxicity of CHB and CBO in human hepatocyte L02 cells as measured by the MTT and RCE assays

The cytotoxicity of CHB and CBO was first examined with the MTT assay. Cells were incubated with different concentrations of CHB and CBO at 37 °C for 1 h. No cytotoxicity was observed for CHB up to 1000 μM (data not shown). However, CBO exhibited cytotoxicity starting from 40 μM, at which the cell viability was 80% ($p < 0.05$). At 100 μM, the highest concentration tested, the cell viability was lowered down to 51% ($p < 0.001$) (Fig. 1).

Long-term survivability of cells was then evaluated by the RCE assay and the results were quite different from those obtained with the MTT assay. For CHB, the survival rate of cells exhibited a statistically significant decrease even at 20 μM and was further decreased with the increase in the concentration. At 500 μM, the RCE value was only 6.3% ($p < 0.001$, Fig. 2A), and at 1000 μM no cells survived at all. On the other hand, CBO showed much higher toxicity. At a concentration as low as 0.2 μM, the survival rate of cells was decreased to 74.9% ($p < 0.001$), and further lowered down with the increase in the concentration. When the CBO concentration reached 10 μM, only few cells survived (Fig. 2B).

The genotoxicity of CHB and CBO as measured by the standard comet assay

A preliminary experiment was carried out to screen the appropriate concentrations of CHB and CBO for comet assays. Five concentrations, which were 10, 50, 200, 500, and 1000 μM, were selected for CHB. However, consistent with the MTT assay results with CBO when 40 μM CBO exhibited cytotoxicity (Fig. 1), use of CBO at 20, 30, 40, and 50 μM resulted in such severe DNA damage that the DNA images were unable to be observed. As a result, the highest used concentration for CBO was set at 10 μM and the actual concentrations chosen for CBO were 0.5, 1, 2, 5, and 10 μM.

Using the standard comet assay, both CHB and CBO were observed to induce concentration-related increases in the rates of DNA migration, indicating that they generated

strand breaks. For CHB, statistically significant increases in the rates of DNA migration were observed at 200, 500, and 1000 μM (Fig. 3A). On the other hand, CBO was much more potent than CHB. Even at a concentration as low as 1 μM, CBO induced a statistically significant increase in the rate of DNA migration ($p < 0.05$, Fig. 3B). The rates of DNA migration at 2, 5, and 10 μM increased with the increases in the concentrations and the values were close to those observed with 200, 500, and 1000 μM CHB, respectively. Therefore, both compounds were capable of inducing strand breakage in cells and were genotoxic.

Determination of the types of DNA breaks induced by CHB and CBO

The above results indicated that CHB and CBO generated strand breaks in cells. However, it was not clear what types of DNA damage CHB and CBO caused. To determine the types of DNA damage, the pH 11.9 and pH 9 comet assays were employed.

The results were shown in Fig. 4. CHB caused an increase in the rate of DNA migration in a concentration-dependent manner with both the pH 11.9 and pH 9 comet assays, although the increase became statistically significant only starting from 500 μM, indicating that CHB generated SSB. However, at the same concentrations, the rates of DNA migration at pH 11.9 were considerably lower than those at pH > 13 (8.1% vs. 22.4% at 500 μM and 14.2% vs. 38.4% at 1000 μM, respectively), indicating that CHB also produced ALS. Thus, the results showed that CHB was capable of generating both SSB and ALS.

Surprisingly, CBO failed to induce an increase in the rate of DNA migration relative to the negative control at all concentrations tested (0.5, 1, 2, 5, and 10 μM) with both the pH 11.9 and pH 9 comet assays, while 200 μM DEB (which was used as the positive control because DEB has been demonstrated to induce SSB (Wen et al., 2011)) caused statistically significant increases in these experiments (data not shown), indicating that CBO at the used concentrations produced only ALS.

Examination of DNA damage caused by CHB and CBO directly on naked DNA

To explore the mechanisms by which the two compounds damaged DNA, the abilities of CHB and CBO to directly induce strand breakage on DNA were investigated. Based on their chemical structures, CBO was expected to readily react with DNA bases to form adducts, whereas the reactivity of CHB was expected to be weaker than that of CBO. If the reactions occur at the *N*7-positions of 2′-deoxyguanosine residues in DNA, the adducts formed would probably undergo spontaneous depurination to generate abasic sites (Gates et al., 2004), i.e., ALS, which can be detected by the comet assay using a strongly alkaline electrophoresis solution.

To investigate damage on naked DNA, a variant of comet assay, which is called the acellular comet assay (Kasamatsu et al., 1996), was employed. In the variant, cells are first lysed and then are exposed to chemicals tested. After lysis, plasma membranes, cytoplasm, nucleoplasm, and almost all histones are removed (Collins, 2004). In this situation the chemicals tested directly interact with naked DNA without any interference by cellular components. Consequently, the results reflect the direct damage caused by the chemicals on DNA.

The results showed that CHB failed to induce an increase in the rate of DNA migration even at the highest concentration tested (1000 μM, data not shown), whereas CBO caused a concentration-dependent increase (Fig. 5). However, the CBO-induced increases in the rates of DNA migration became statistically significant only at the two higher concentrations (5 and 10 μM), and the net increases relative to the control were considerably smaller than those observed with the standard comet assay (Fig. 3B). Therefore, the results indicated that

CHB was unable to directly cause strand breakage on naked DNA, whereas CBO was capable of generating strand breaks (SSB and/or ALS) likely through chemical mechanisms.

Examination of cross-linking caused by CHB and CBO

We have demonstrated in the preceding paper that CBO could cause the formation of cross-links of globin (Elfarra and Zhang, 2012). Thus, it was logical to investigate if CBO could induce DNA cross-linking. In this experiment, MMS was employed to make additional DNA damage and DEB (1000 μM) was used as the positive control, because DEB at this concentration induced strong DNA cross-linking as demonstrated in our previous study (Wen et al., 2011).

The result showed that at the concentration tested (1, 2, 5, and 10 μM), CBO did not cause a decrease in the rate of DNA migration (data not shown). By contrast, DEB caused a large decrease in the rate of DNA migration (8.1% vs. 69.2% for the control, $p < 0.001$), clearly demonstrating the cross-linking ability of DEB. Based on its chemical structure, CHB was not expected to possess the cross-linking capability. Nonetheless, CHB was still examined similarly. As expected, CHB did not show any DNA migration-reducing effect. Therefore, both CHB and CBO did not induce DNA cross-linking as examined by the comet assay.

Mutagenicity of CHB and CBO examined using the Ames test

As shown in Fig. 6, CHB resulted in a dose-related increase in the number of revertant colonies from 0.5 to 5 mg per plate over the control in the strain TA1535. As positive controls, sodium azide at 2 μg per plate and EB at 5 mg per plate caused 33- (data not shown) and 4.4-fold increases, respectively. All the increases were statistically significant. CHB at the lowest dose tested (0.5 mg per plate) induced a 2.3-fold increase, which was slightly lower than three-fold, the cut-off value between a mutagenic and non-mutagenic response. However, starting from the dose at 1 mg per plate, all doses tested showed three-fold and larger increases in the numbers of revertant colonies. Together with the dose-response relationship, the data indicated that CHB gave a positive result in the Ames test, that is to say, CHB is a mutagen.

However, in the strain TA1537, CHB did not cause increases in the numbers of revertant colonies over the control at 0.5, 1, and 2 mg per plate, and exhibited toxicity at 4 and 5 mg per plate (data not shown), suggesting that CHB did not induce frameshift mutation in this bacterial strain.

CBO exhibited only toxicity in the bacterial strain TA1535 when it was used at levels in between 50 μg to 2 mg per plate. It also caused toxicity in the TA1537 strain when used at levels in between 10 μg to 2 mg per plate. At lower levels (5, 10, and 25 μg per plate for TA1535, and 5 μg per plate for TA1537), no increases in the numbers of revertant colonies were observed (data not shown). Thus, CBO is a highly toxic compound in the two used bacterial strains and this toxicity prevented a reliable assessment of its mutagenicity in these two bacterial strains.

Discussion

CHB and CBO are two known in vitro metabolites of BD that may also be formed in vivo in immune and bone marrow cells. Studies on their cytotoxicity, genotoxicity, and mutagenicity will help determine the potential toxicological significance of the two compounds, providing grounds for further investigation. CHB and CBO were expected to be cytotoxic, genotoxic, and probably mutagenic due to their reactivity towards nucleophiles. The chloromethyl moiety of CHB is expected to have moderate reactivity towards biological

nucleophiles; CBO is a strong Michael acceptor with its two ends being much more reactive than the chloromethyl moiety of CHB (Elfarra and Zhang, 2012).

Indeed, the experimental results were consistent with the expectations. Both compounds were found to be cytotoxic and genotoxic with CBO being consistently more potent than CHB. Quantitatively, in terms of the cytotoxicity as measured using the RCE assay and the genotoxicity as determined with the standard comet assay, CBO was approximately 100-fold more potent than CHB, because 100-fold higher concentrations of CHB were needed to produce the same effects as those caused by CBO at a given concentration. For instance, the RCE value at 1 μM CBO was 53.0%, and 100 μM CHB gave a similar value at 48.4%. Using the standard comet assay, the rates of DNA migration were 14.7, 25.2, and 34.6% for 2, 5, and 10 μM CBO, respectively, and the values were 13.9, 22.4, and 38.4% for 200, 500, and 1000 μM CHB, respectively.

There clearly existed differences in genotoxicity between the two compounds. CHB failed to directly generate breaks (SSB and/or ALS) on naked DNA, whereas CBO could do so, probably due to the higher reactivity of CBO towards DNA over CHB (Zeng et al., manuscript in preparation). In addition, CHB induced both SSB and ALS in living cells, whereas CBO produced only ALS. These results suggest the involvement of cellular components in modulating the genotoxicity of these two compounds.

CHB was capable of generating SSB and ALS in living cells, however, it caused neither SSB nor ALS on naked DNA, suggesting that the genotoxicity of CHB was mediated by one or more bioactivation products and/or through some unknown cellular mechanisms. On the other hand, CBO produced ALS in living cells and could also cause breaks (SSB and/or ALS) directly on naked DNA. However, the amounts of the breaks caused on naked DNA were considerably smaller than those formed in living cells at a given concentration, because the net increases in the rates of DNA migration relative to the control in living cells were significantly greater than those in lysed cells at the same CBO concentrations. Considering that CBO has high reactivity and readily reacts with GSH, and because the highest concentration of CBO used in our experiments was only 10 μM whereas the intracellular GSH concentration is usually at a much higher level (mM level), it was reasonable to speculate that CBO would be completely scavenged by intracellular GSH before it could react with DNA. However, as demonstrated with GSH conjugates of other Michael acceptors, retro-Michael reactions could occur spontaneously or through catalysis by GSH *S*-transferases to free CBO to react with DNA and cause genotoxicity (Chen and Armstrong, 1995; Alary et al., 2003). The genotoxicity of CBO with naked DNA and its reactivity with DNA bases provide evidence for this hypothesis.

The DNA cross-linking potential of CBO was examined because of the previously demonstrated cross-linking ability of CBO toward hemoglobin (Elfarra and Zhang, 2012). Interestingly, CBO did not result in DNA cross-linking as evaluated by the comet assay, possibly due to lower reactivity of CBO towards nucleophilic amino groups and nitrogen atoms in DNA in comparison with CBO reactivity towards sulfhydryl groups in proteins.

Mutagenicity of CHB and CBO was also examined and the results showed that whereas CHB was mutagenic, the severe toxicity of CBO in the used bacterial strains prevented a reliable assessment of its mutagenicity. As a strong Michael acceptor, CBO can react with proteins, lipids, DNA, and other biomolecules. These reactions might cause havoc in cells and lead to cell death. Similarly, hydroxymethyl vinyl ketone, a structural analog of CBO and also a reactive Michael acceptor (Krause et al., 2001), was also observed to be highly toxic in the Ames test and its mutagenicity could not be assessed due to its toxicity (data not shown). Future studies with alternative bacterial strains, such as TA98 and TA100, could

allow for more definitive conclusions to be made regarding the relative roles of point- and frameshift mutations in CHB mutagenicity.

The results obtained in the present study clearly demonstrated the toxic and mutagenic potential of CHB and CBO. The two compounds exhibited different characteristics in genotoxicity and mutagenicity and may have different significance in the mutagenicity and/or carcinogenicity of BD. CHB displayed weaker cytotoxicity and genotoxicity, however, it was mutagenic. Thus, CHB may have carcinogenic potential, similar to other chlorohydrins that have been associated with leukemia and pancreatic cancers (Greenberg et al., 1990), and lymphatic and hematopoietic cancers (Ott et al., 1989; Benson and Teta, 1993). In this regard, ethylene chlorohydrin, propylene chlorohydrin, and 3-chloro-1,2-propanediol, are considered to be potential carcinogens (Laskin et al., 1980; Kluwe et al., 1983; Ott et al., 1989; Greenberg et al., 1990; Benson and Teta, 1993; Lee et al., 2005; Skamarauskas et al., 2007). Interestingly, CBO was a much more potent genotoxicant by the RCE assay and the comet assay than CHB. It caused a statistically significant decrease in the long-term survivability of cells at 0.2 μM as tested with the RCE assay and produced a statistically significant increase in the amount of DNA breaks at 1 μM as examined with the standard comet assay. By comparison, using the same cell line and methods, the data for DEB, which is generally considered the ultimate culprit for mutagenicity/carcinogenicity of BD due to its strong reactivity and cross-linking ability (Boogaard and Bond, 1996), were 30 and 50 μM, respectively (Wen et al., 2011). Obviously, in terms of the RCE assay and comet assay endpoints, CBO was a much more potent genotoxicant than DEB. Thus, even a small amount of CBO produced in vivo may have significant genotoxicity and could greatly affect risk assessment for humans exposed to BD. Hence, additional studies are warranted to characterize the roles of these metabolites in the in vivo metabolism and toxicity of BD.

Acknowledgments

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Abbreviations

ALS	alkali-labile sites
BD	1,3-butadiene
CBO	1-chloro-3-buten-2-one
CHB	1-chloro-2-hydroxy-3-butene
DEB	1,2,3,4-diepoxybutane
EB	3,4-epoxy-1-butene
EBD	3,4-epoxy-1,2-butanediol
FBS	fetal bovine serum
GSH	glutathione
MMS	methyl methanesulfonate
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide
RCE	relative cloning efficiency
S.D	standard deviation

SSB	single-strand breaks
%Tail DNA	percentage of DNA in the tail

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Highlights

- 1-Chloro-2-hydroxy-3-butene (CHB) is cytotoxic and genotoxic in human liver cells
- The CHB metabolite, 1-chloro-3-buten-2-one (CBO) is ~100-fold more toxic than CHB
- CHB and CBO cause DNA alkali-labile sites, but only CBO directly causes DNA breaks
- CHB is mutagenic in the Ames test, but CBO is too toxic in the assay
- The results suggest a role for CHB in 1,3-butadiene genotoxicity and mutagenicity

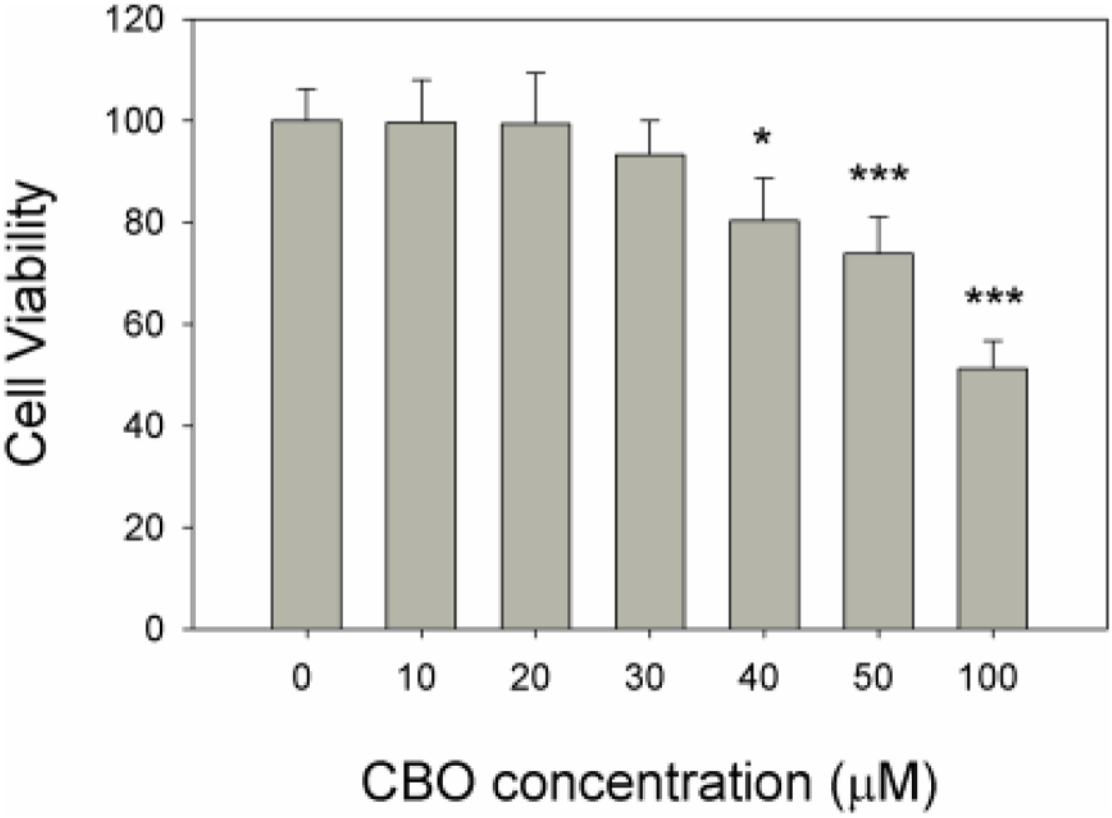

Fig. 1.

Cytotoxicity of CBO on human hepatocyte L02 cells as measured by the MTT assay. Cells were exposed to CBO in FBS-free media at 37 °C for 1 h. Results are shown as the mean ± SD of **the values obtained from** six independent samples (*$p \leq 0.05$, ***$p \leq 0.001$).

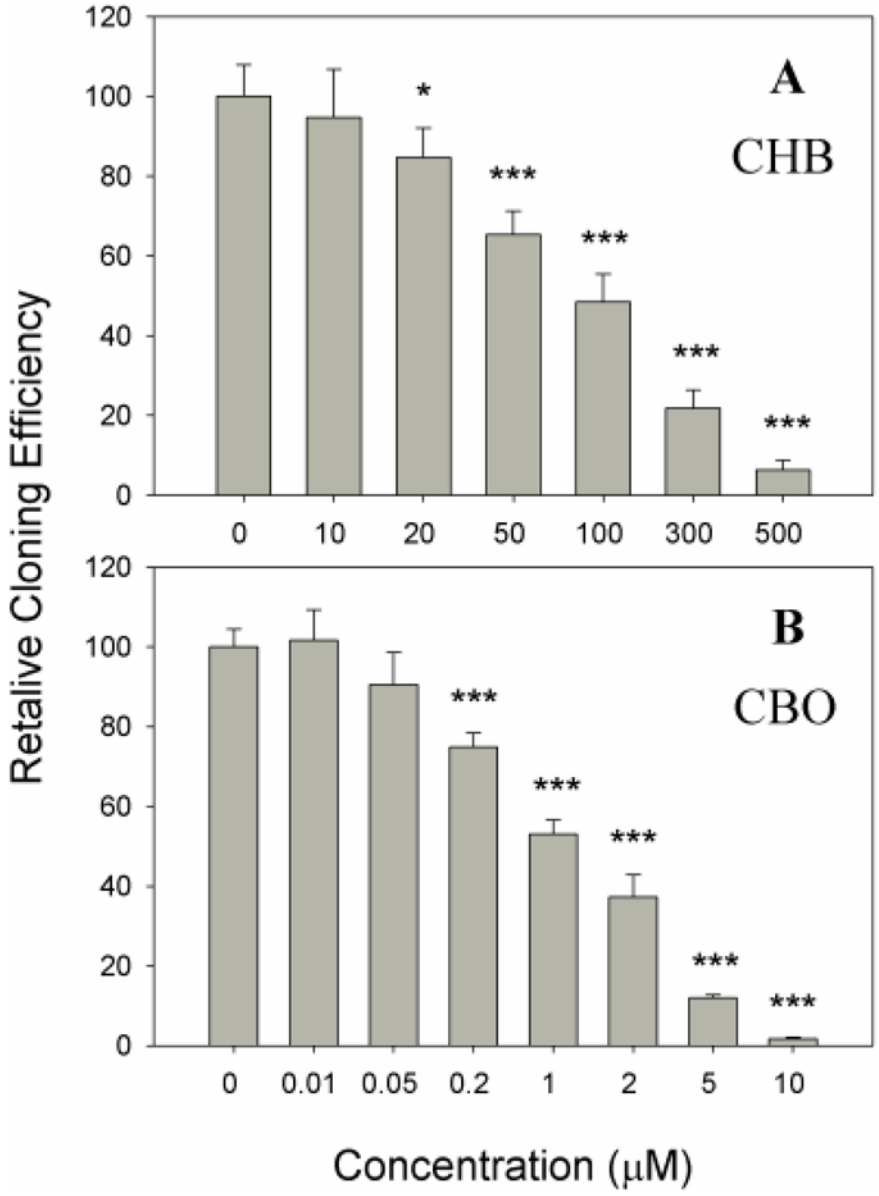

Fig. 2.

The effects of CHB (**A**) and CBO (**B**) on the long-term survival (relative cloning efficiency) of human hepatocyte L02 cells. Cells were exposed to CHB and CBO in FBS-free media at 37 °C for 1 h. Results are shown as the mean ± SD of **the values obtained from** three independent samples (*$p \leq 0.05$, ***$p \leq 0.001$).

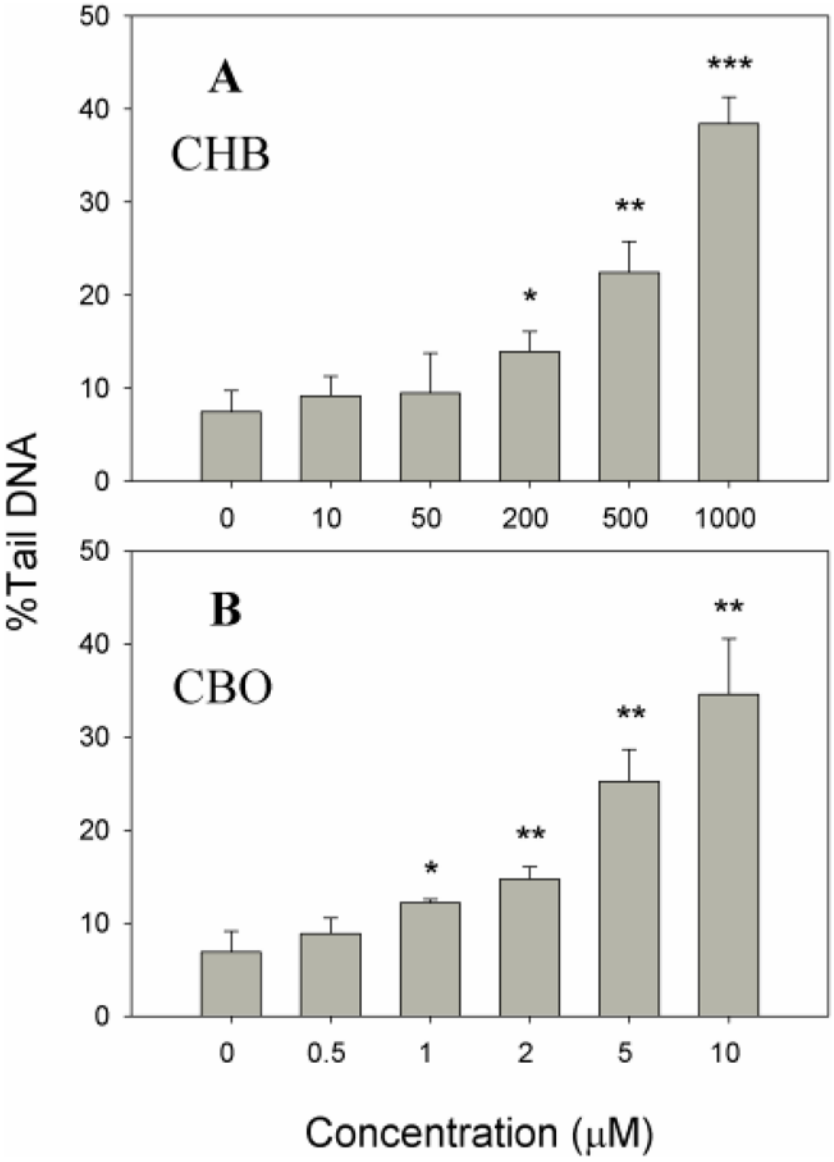

Fig. 3.

DNA migration caused by different concentrations of CHB (**A**) and CBO (**B**) on human hepatocyte L02 cells as determined by the standard comet assay. Cells were exposed to CHB and CBO in FBS-free media at 37 °C for 1 h. Results are shown as the mean ± SD of **the values obtained from** three independent samples (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

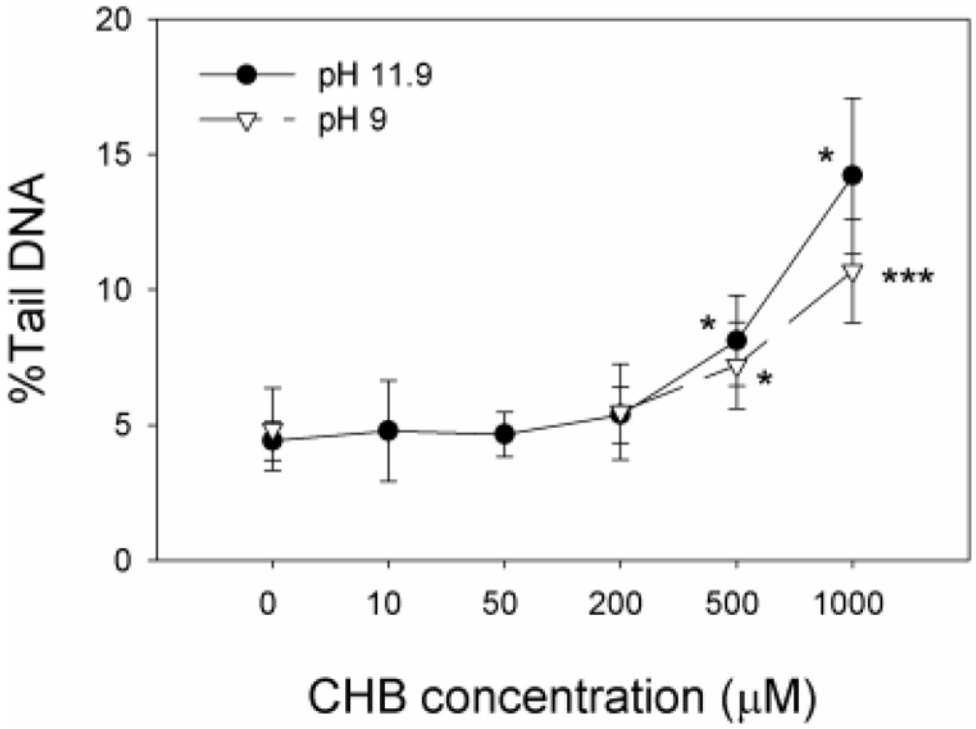

Fig. 4.

DNA migration caused by different concentrations of CHB in human hepatocyte L02 cells as determined by the pH 11.9 and pH 9 comet assays to distinguish SSB from ALS. Cells were exposed to CHB in FBS-free media at 37 °C for 1 h. Results are shown as the mean ± SD of **the values obtained from** three independent samples (*$p \leq 0.05$, ***$p \leq 0.001$).

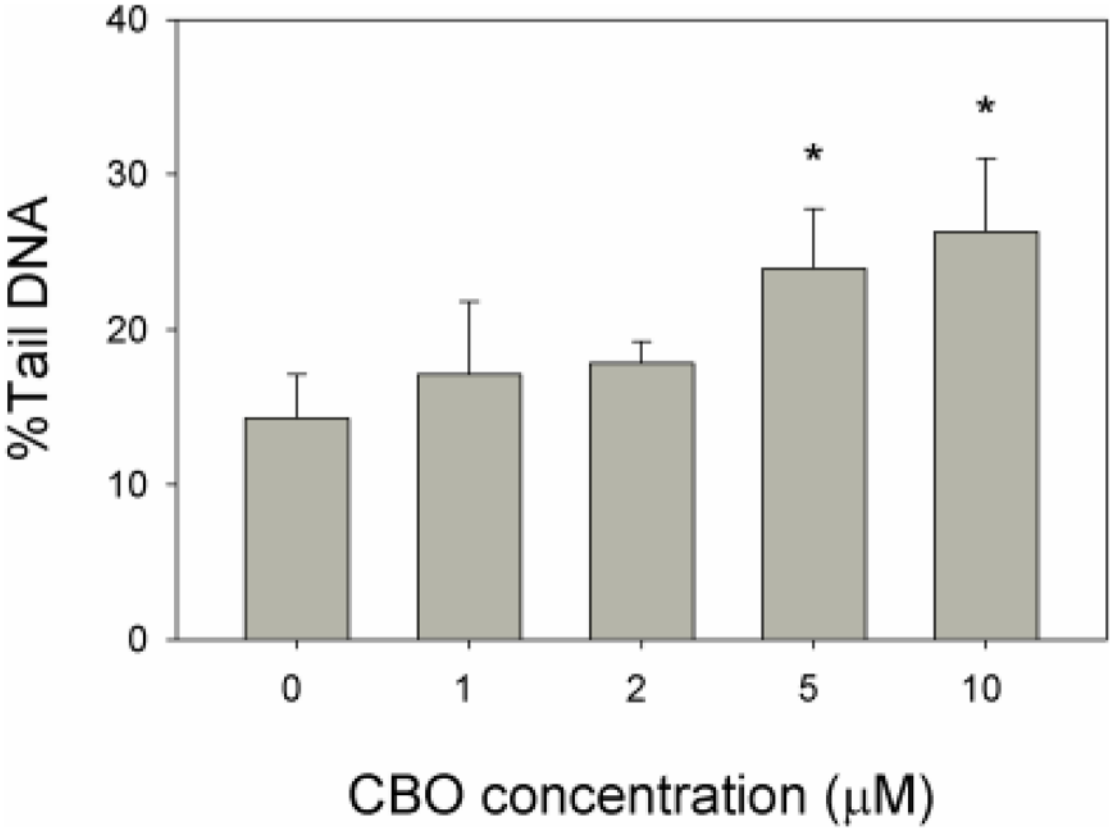

Fig. 5.

DNA migration caused by different concentrations of CBO on lysed cells as determined by the acellular comet assay. Cells were first lysed and then exposed to CBO in 400 mM pH 7 phosphate buffer at 37 °C for 1 h. Results are shown as the mean ± SD of **the values obtained from** three independent samples (*$p \leq 0.05$).

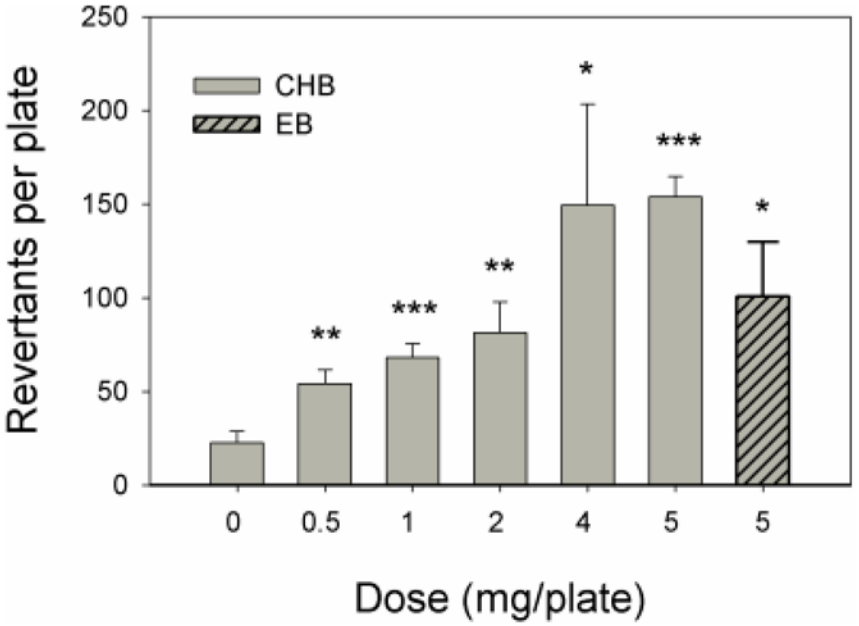

Fig. 6.

The numbers of revertant colonies per plate observed for strain *Salmonella* TA1535 after being treated with different doses of CHB as described in the Materials and methods section. EB (5 mg per plate) was used as a positive control. Results are shown as the mean ± SD of **the values obtained from** three independent samples (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).