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Agents of earthy-musty taste and odor in water: Evaluation of cytotoxicity, genotoxicity and toxicogenomics

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HIGHLIGHTS

- Biological effects of 2-MIB and GEO were investigated in human cells.
- 2-MIB and GEO were cytotoxic.
- At non-cytotoxic concentrations 2-MIB and GEO were not genotoxic.
- 2-MIB and GEO did not alter the expression of DNA damage responsive genes.

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ABSTRACT

Considering the limited number of studies on the biological effects on human health of cyanobacterial compounds that cause taste and odor, the present study assessed the cytotoxic and genotoxic potentials of 2-methylisoborneol (2-MIB) and geosmin (GEO) using the MTT assay and the *in vitro* comet and cytokinesis-block micronucleus (CBMN-Cyt) assays in human HepG2 cells. The toxicogenomics of genes responsive to DNA damage and metabolization by the exposure of cells to 2-MIB and GEO were also investigated. The results showed that concentrations of 2-MIB and GEO above 100 and 75 μg/mL, respectively, were cytotoxic to HepG2 cells. Doses of 2-MIB (12.5, 25, 50, 75 and 100 μg/mL) and GEO (12.5, 25, 50, and 75 μg/mL) were unable to induce neither DNA damage nor events associated with chromosomal instability. Similarly, no concentration of each compound induced increments in the expression of *CDKN1A*, *GADD45α*, *MDM2* and *TP53* DNA damage responsive genes as well as in *CYP1A1* and *CYP1A2* metabolizing genes. Although cytotoxicity was observed, concentrations that caused it are much higher than those expected to occur in aquatic environments. Thus, environmentally relevant concentrations of both compounds are not expected to exhibit cytotoxicity or genotoxicity to humans.

1. Introduction

Among the numerous terpenoid compounds found in the environment, 2-methylisoborneol (2-MIB) and geosmin (GEO) are widely known for their odorous and volatile properties (Gerber and Lecheval, 1965; Wang et al., 2011). They are synthesized and secreted as secondary metabolites by microorganisms such as cyanobacteria (Jüttner and Watson, 2007), as a consequence of the eutrophication process caused by anthropic activities such as domestic and industrial sewage discharges in natural waters (Codd, 2000). The presence of 2-MIB and GEO in public water supplies is the main cause of the earthy-musty taste and odor that renders drinking water unpalatable (Wnorowski, 1992; Ho et al., 2012), and these compounds are recalcitrant to conventional water treatment (Rittmann et al., 1995; Nerenberg et al., 2000). Studies have detected 2-MIB and GEO in various worldwide sources of raw water with concentration range of 0–800 ng/L (Bendati et al., 2005; Izaguirre and Taylor, 2007; Proulx et al., 2012; Qi et al., 2012). Hence, no definitive pathways have been elucidated for the biodegradation of 2-MIB and GEO (Ho et al., 2012).

Studies on the biological effects of *in vivo* and *in vitro* exposures to cyanobacteria metabolites have been focused on the bioactivity of

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toxins (cyanotoxins) (Zegura et al., 2011). In fact, a diverse range of cyanotoxins have been investigated concerning their carcinogenic and mutagenic potentials, such as microcystins (Ohta et al., 1992; Zegura et al., 2004, 2006), nodularins (Ohta et al., 1994; Fessard et al., 2004; Maatouk et al., 2004), cylindrospermopsin (Falconer and Humpage, 2001; Maire et al., 2010; Straser et al., 2011), and anatoxin-a (Repavich et al., 1990).

To our knowledge, mutagenic activity of 2-MIB and GEO was only investigated using the Ames test, with both compounds leading to negative results to exposed *Salmonella typhimurium* tester strains (Masaki et al., 1987; Dionigi et al., 1993). However, 2-MIB and GEO were toxic to *S. typhimurium* tester strains in the Ames test (Dionigi et al., 1993) and inhibited the early development of sea urchin embryos, presenting IC_{50} (50% inhibitory concentration) toxicity values similar to that observed for the *S. typhimurium* strains in the Ames test (Nakajima et al., 1996). Thus, considering this scenario, studies about the biological effects of 2-MIB and GEO are urgently needed to fully evaluate the safety of drinking water and to assess the risk associated with exposure to these compounds for human health.

The aim of this study was to investigate the cytotoxic and genotoxic activities of 2-MIB and GEO in the metabolically active human hepatoma cell line, HepG2, which retains the activities of inducible phase I and phase II xenobiotic metabolizing enzymes (Knasmüller et al., 1998, 2004). In addition, we have analyzed DNA damage and chromosomal instability using the comet assay and the cytokinesis blocked micronucleus cytome (CBMN-Cyt) assays, respectively. The 2-MIB and GEO mediated modulation of the expression of genes involved in DNA damage as well as cytochrome P450 responses was also assessed.

2. Material and methods

2.1. 2-MIB and GEO

2-MIB (CAS 2371-42-8) and GEO (CAS 16423-19-1) were purchased from Wako Chemicals USA, Inc., 1600 Bellwood Rd., Richmond, VA 23237. The chemical structures of 2-MIB and GEO are presented in Fig. 1. Solutions of 2-MIB and GEO were made using methanol 0.5%. The chemical mutagen benzo[a]pyrene (BaP) (CAS 50-32-8) was purchased from Sigma-Aldrich, St. Louis, MO, USA and used as positive control. All dilutions were prepared immediately before use.

2.2. Cell line and culture conditions

HepG2 cells were purchased from Rio de Janeiro Cell Bank (catalogue 0103). HepG2 cells were maintained as a monolayer in 75 cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen) and kept in a humidified 5% CO_2 atmosphere at a temperature of 37 °C until confluence was reached.

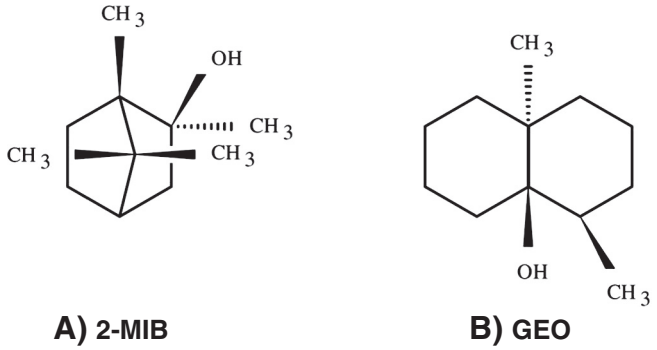

Fig. 1. Chemical structures of 2-methylisoborneol (2-MIB) $C_{11}H_{20}O$ and geosmin (GEO) $C_{12}H_{22}O$ (Ho et al., 2012).

2.3. MTT assay

The mitochondria dependent reduction of MTT (3-(4,5-dimethylthiazol-2yl) 2,5-diphenyl-tetrazolium bromide) to formazan was used to determine cytotoxicity. Cells were seeded in a 96-well plate, cultured for 24 h and treated on the following day with 2-MIB and GEO at concentrations of 25, 50, 75, 100, 200 and 400 μg/mL for 24 h. MTT solution (5 mg/mL) was added to each well and incubated at 37 °C for 2 h. The formazan crystals that formed were dissolved in 100 μL DMSO for 5 min under shaking. Each plate was read immediately on a microplate reader (Thermo Scientific, USA) at a wavelength of 570 nm. Three independent experiments, in triplicate, were performed for each compound.

2.4. The cytokinesis-block micronucleus (CBMN) cytome assay

The CBMN cytome (CBMN-Cyt) assay was carried out according to Natarajan and Darroudi (1991) and Fenech (2007) with slight modifications. Briefly, HepG2 cells were seeded on 24-well plates at density of 1×10^5 and incubated for 20 h at 37 °C and 5% CO_2. They were then exposed to 12.5, 25, 50, 75 and 100 μg/mL of 2-MIB and 12.5, 25, 50 and 75 μg/mL of GEO for 24 h. BaP (30 μM) was used as positive control and 0.5% methanol diluted in DMEM as the negative control. After treatment, cells were washed twice in Dulbecco's phosphate-buffered saline (DPBS), and cytochalasin B (Cyt B; Sigma) was added to a final concentration of 5 μg/mL in complete fresh medium. Twenty-eight hours later, Cyt B was removed and the cells were washed twice with DPBS at 37 °C, trypsinized with 350 μL trypsin (Invitrogen) and resuspended with complete medium. The cells were harvested by cytocentrifugation (Cientec). Then, 100–120 μL of cell suspension was transferred to cytocentrifuge cups and centrifuged for 5 min at 151 ×*g* to produce 1 spot per slide. Slides were removed, fixed and stained with Instant Prov (Newprov®). After staining, slides were air dried and examined under 1000× magnification using a light microscope. Three independent experiments, in duplicate, were performed for each compound.

Micronuclei (MNi), nuclear buds (NBUDs), and nucleoplasmatic bridges (NPBs) were counted in 1000 binucleated cells (BNC) per experimental point and were scored according to Fenech (2000). The nuclear division cytotoxicity index (NDCI) was estimated by scoring 500 cells with one to four nuclei, apoptotic and necrotic. The NDCI was calculated using the formula [Ap + Nec + M1 + 2(M2) + 3(M3) + 4(M4)] / 500, where M1–M4 represent the number of cells with one to four nuclei, respectively, Ap = number of apoptotic cells, and Nec = number of necrotic cells. Analysis of variance (one-way ANOVA) with Dunnett's *post hoc* test at $P < 0.05$ was used to quantitatively determine the difference between negative control and treated groups.

2.5. The single cell microgel electrophoresis assay

HepG2 cells were seeded at a density of 1×10^4 on 96-well plates and incubated for 20 h. Then, cells were exposed to concentrations of 12.5, 25, 50, 75 and 100 μg/mL of 2-MIB and 12.5, 25, 50 and 75 μg/mL of GEO for 4 and 24 h. Positive (BaP 30 μM) and negative (methanol 0.5%) controls were included. After treatments, the comet assay was performed according to Tice et al. (2000) with minor modifications. Briefly, a base layer of 1.5% normal-melting agarose (CAS:9012-36-6, Invitrogen) was placed on a microscope slide and 40 μL of HepG2 test cells suspended in 140 μL 0.5% low-melting agarose (CAS:9012-36-6, Invitrogen) at 37 °C was then spread over the base layer. A coverslip was added and agarose was allowed to solidify at 4 °C for 15 min. After agarose solidification, the coverslip was removed and the slides were immersed into a lysis solution (89 mL stock solution; 10 mL