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Concentration- and time-dependent genotoxicity profiles of isoprene monoepoxides and diepoxide, and the cross-linking potential of isoprene diepoxide in cells

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ABSTRACT

Isoprene, a possible carcinogen, is a petrochemical and a natural product being primarily produced by plants. It is biotransformed to 2-ethenyl-2-methyloxirane (IP-1,2-O) and 2-(1-methylethenyl)oxirane (IP-3,4-O), both of which can be further metabolized to 2-methyl-2,2′-bioxirane (MBO). MBO is mutagenic, but IP-1,2-O and IP-3,4-O are not. While IP-1,2-O has been reported being genotoxic, the genotoxicity of IP-3,4-O and MBO, and the cross-linking potential of MBO have not been examined. In the present study, we used the comet assay to investigate the concentration- and time-dependent genotoxicity profiles of the three metabolites and the cross-linking potential of MBO in human hepatocyte L02 cells. For the incubation time of 1 h, all metabolites showed positive concentration-dependent profiles with a potency rank order of IP-3,4-O > MBO > IP-1,2-O. In human hepatocellular carcinoma (HepG2) and human leukemia (HL60) cells, IP-3,4-O was still more potent in inducing DNA breaks than MBO at high concentrations (>200 μM), although at low concentrations (≤200 μM) IP-3,4-O exhibited slightly lower or similar potency to MBO. Interestingly, their time-dependent genotoxicity profiles (0.5–4 h) in L02 cells were different from each other: IP-1,2-O and MBO (200 μM) exhibited negative and positive profiles, respectively, with IP-3,4-O lying in between, namely, IP-3,4-O-caused DNA breaks did not change over the exposure time. Further experiments demonstrated that hydrolysis of IP-1,2-O contributed to the negative profile and MBO induced cross-links at high concentrations and long incubation times. Collectively, the results suggested that IP-3,4-O might play a significant role in the toxicity of isoprene.

Abbreviations: DEB, 1,2,3,4-diepoxybutane; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HepG2, human hepatocellular carcinoma cells; HL60, human leukemia cells; IMDM, Iscove's Modified Dulbecco's Medium; IP-1,2-O, 2-ethenyl-2-methyloxirane; IP-3,4-O, 2-(1-methylethenyl)oxirane; MBO, 2-methyl-2,2′-bioxirane; mCPBA, *m*-chloroperoxybenzoic acid; MMS, methyl methanesulfonate; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; PBMCs, peripheral blood mononuclear cells; SD, standard deviation; %Tail DNA, percentage of DNA in the tail.

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1. Introduction

Isoprene (2-methyl-1,3-butadiene), the 2-methyl analog of human carcinogen 1,3-butadiene, is an important petrochemical that is primarily used in the manufacture of synthetic rubber. It is also a natural product that is produced by plants, animals [1,2], bacteria [3], and humans [4,5].

Isoprene is a possible carcinogen. Animal toxicology studies have indicated that it is carcinogenic to mice [6] but is very weakly carcinogenic to rats [7]. Isoprene is classified as "possibly carcinogenic to humans (group 2B)" by the International Agency for Research on Cancer [8] and as "reasonably anticipated to be a human carcinogen" by the U.S. National Toxicology Program [9]. However, it has not been classified as a human carcinogen due to lack of epidemiological data.

The environmental sources of isoprene include natural and anthropogenic ones. Emissions from plants are the primary source of isoprene in the atmosphere; the quantities of emissions from plants exceed those produced synthetically by approximately 300-fold [2]. In fact, isoprene is the single largest biogenic nonmethane hydrocarbon emitted into the Earth's atmosphere; the annual global emission is estimated to be $\sim 6 \times 10^{11}$ kg [10]. The major anthropogenic sources are combustion processes, including biomass burning, tobacco smoking, and automobile exhaust [9,11,12]. Thus, like 1,3-butadiene, isoprene is also ubiquitous in the environment; its concentration in U.S. ambient air ranges from 1 to 21 ppb and generally is less than 10 ppb [9].

Human exposure to isoprene is largely caused by its generation through endogenous processes, because it is the major endogenously produced hydrocarbon [1] and is abundant in human breath at concentrations in the range of 50–1000 ppb [13]. Nonetheless, smoking significantly increases human exposure to isoprene because tobacco smoke is the primary source of isoprene in indoor air [9,14]. Isoprene is one of the major hazardous volatile organic compounds in cigarette smoke; its total yield (~800 μg/cigarette) is the second largest among 14 hazardous volatile organic compounds [12]. It is ranked third (after 1,3-butadiene and acetaldehyde) with respect to cancer hazards stemming from smoking by the World Health Organization on the basis of its abundance in cigarette smoke and its animal carcinogenicity [15].

Similarly to 1,3-butadiene, isoprene undergoes oxidative metabolism, which is primarily mediated by cytochrome P450 2E1, followed by P450 2B6 [16], to produce two isomeric monoepoxides, 2-ethenyl-2-methyloxirane (i.e., isoprene-1,2-oxide, IP-1,2-O) and 2-(1-methylethenyl)oxirane (i.e., isoprene-3,4-oxide, IP-3,4-O). Both monoepoxides can be further metabolized to the diepoxide, 2-methyl-2,2′-bioxirane (MBO) (Fig. 1) [16–19]. The epoxides can be hydrolyzed by epoxide hydrolase to form the corresponding diols or epoxydiols, or can be conjugated with glutathione [16,20,21]. For the two monoepoxides, IP-1,2-O is the major metabolite and IP-3,4-O is the minor one (~20%) [16–18].

Isoprene itself is not mutagenic as examined by the Ames test, even after metabolic activation using rat liver microsomes [22]. Unlike the monoepoxide of

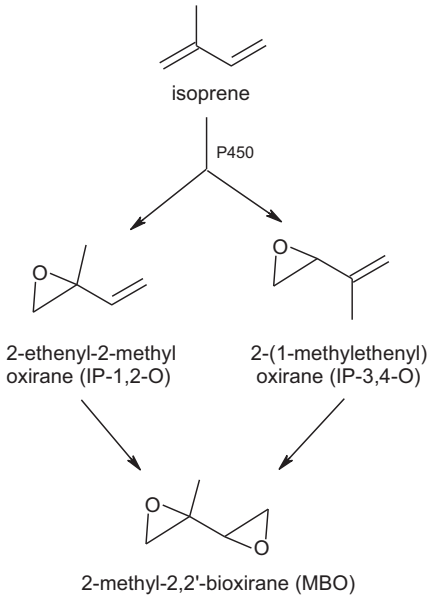

Fig. 1. The metabolism pathways of isoprene.

1,3-butadiene, IP-1,2-O and IP-3,4-O are non-mutagenic [23,24]. However, MBO was found to be as mutagenic as 1,2,3,4-diepoxybutane (DEB) [23,24], the diepoxide of 1,3-butadiene. On the other hand, isoprene itself did not induce strand breaks in the absence of metabolic activation but did so with metabolic activation in peripheral blood mononuclear cells (PBMCs) and human leukemia cells (HL60) as evaluated by the comet assay [25]. This may indicate that isoprene metabolites are genotoxic. Indeed, IP-1,2-O has been found to be genotoxic in PBMCs and HL60 [25,26]. However, so far the genotoxicity of IP-3,4-O and MBO has not been examined yet.

In comparison with 1,3-butadiene, the presence of the extra methyl group in isoprene has a profound influence on properties of its metabolites, including reactivity, mutagenicity, etc., because the methyl group causes steric hindrance and also introduces an asymmetric factor in these molecules. Due to the asymmetry, there exist two monoepoxides that show quite distinct reactivity in some reactions. For instance, IP-1,2-O is easily hydrolyzed, whereas IP-3,4-O (and also MBO) is much more resistant to hydrolysis. Their half-lives at physiological pH and temperature are 1.25 and 73 h (46 h for MBO), respectively [23,24]. The difference between IP-1,2-O and IP-3,4-O is especially great in the acid-catalyzed hydrolysis; the hydrolysis rate constant of IP-1,2-O is 10,000-fold larger than that of IP-3,4-O [27]. For MBO, the asymmetry renders the reactivity of the two oxirane rings different, which was thought to result in suppressed cross-linking potential of MBO compared to DEB (however, the studies were conducted through the reactions between MBO and model compounds (valine methyl ester or purines) and the cross-linking potential of MBO has not been examined in cells or in vivo) [2].

Due to the presence of reactive oxirane moieties in these molecules, IP-1,2-O and IP-3,4-O are alkylating agents

and can react with nucleobases. Their reactions with 2′-deoxyguanosine and 2′-deoxyadenosine under in vitro physiological conditions have been reported [28,29]. They can also react with *N*-terminal valine in hemoglobin to form adducts [30]. The reactivity of these epoxides toward nucleobases may be the molecular basis for their mutagenicity and genotoxicity.

In the present study, we set out to investigate the genotoxic potential of IP-1,2-O, IP-3,4-O, and MBO, and also the cross-linking one of MBO in human hepatocyte L02 cells. Since the genotoxic and cross-linking potential of a chemical depends on its concentration and also exposure time, we investigated the concentration- and time-dependent genotoxicity profiles of IP-1,2-O, IP-3,4-O, and MBO, and the concentration- and time-dependent cross-linking profiles of MBO. Because the genotoxicity of IP-3,4-O was unusually high, we also examined the concentration-dependent profiles of all three metabolites in human hepatocellular carcinoma (HepG2) and human leukemia (HL60) cells to confirm it. The selected experimental means was the comet assay, i.e., single cell gel electrophoresis (SCGE), which is a standard technique to test the genotoxic potential of chemicals. Comet assay has been widely used in biomonitoring of human populations, molecular epidemiology, and assessment of DNA damage/repair and oxidative stress [31–33]. It detects DNA strand breaks through measuring the rate of DNA migration in agarose gel and is capable of examining the presence of cross-links by using a second genotoxicant [31].

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM), Iscove's Modified Dulbecco's Medium (IMDM), and fetal bovine serum (FBS) were obtained from Life Technologies (GIBCO®) (Grand Island, NY, US). Isoprene was obtained from TCI Shanghai (Shanghai, China). IP-1,2-O (CAS# 1838-94-4, 97%) and *m*-chloroperbenzoic acid (mCPBA, 70–75%) were purchased from Alfa Aesar (Ward Hill, MA, US). IP-3,4-O (CAS# 7437-61-8, Cat. No. C7500, purity ≥95%) was obtained from Cheminstock Ltd. (Shanghai, China, http://www.chem-in-stock.com). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), Triton X-100, agarose, propidium iodide, and methyl methanesulfonate (MMS) were purchased from Sigma–Aldrich (St. Louis, MO, US). Other reagents, which were of analytical reagent grade, were obtained from Sinopharm Chemical Reagent Company (Shanghai, China). IP-1,2-O, IP-3,4-O, and MBO were dissolved in dimethyl sulfoxide (DMSO) to prepare the stock solutions. The working solutions, which were prepared just before experiments to avoid hydrolysis, were obtained by diluting the stock solutions by 1000-fold with DMEM or IMDM.

MBO (the mixture of four optical isomers, CAS# 6341-85-1) was synthesized from isoprene and mCPBA based on a procedure described in the literature with a few modifications [19]. Briefly, isoprene (6.8 g, 100 mmol) was dissolved in 550 mL of dichloromethane in an ice-water bath and mCPBA (51.8 g, 70–75%, 220 mmol) was added in portions. The ice-water bath was removed and the mixture was stirred at ambient temperature for 24 h. The suspension was then left in a freezer for 1 h and filtered. The filtrate was washed twice with 5% Na_2SO_3 solution and then twice with saturated Na_2CO_3 solution, and dried over Na_2SO_4. After solvent was removed, MBO (4.3 g, yield 43%) was obtained. Pure MBO (~100% based on the ^{1}H NMR spectrum) was obtained through distillation under water pump pressure (45 °C/32 mmHg). The ^{1}H NMR data, which showed that the obtained MBO consisted of two isomers with a ratio of 3:2, were well consistent with those reported by Bogaards et al. [16].

2.2. Cell culture

The immortalized human normal hepatocyte cell line L02, human hepatocellular carcinoma cell line (HepG2), and human leukemia cell line (HL60) were obtained from the Center of Cell Resources of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The L02 cell line has been used in many studies to examine the biological effects of xenobiotics and investigate the intracellular signaling pathways (see references cited in our previous work [34] and also other papers from our laboratory [35,36]). Isoprene is primarily metabolized in liver, it is thus reasonable to use liver cell lines to investigate the genotoxicity of isoprene metabolites. In addition, we have investigated the genotoxicity of 1,3-butadiene metabolites on the L02 cell line [34,37,38], therefore, using the L02 cell line will facilitate comparison of the results between 1,3-butadiene metabolites and isoprene ones.

L02 and HepG2 cells were cultured as monolayers in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified incubator at 37 °C in 5% CO_2 and routinely passaged by trypsinization when nearly confluent. When cells grew to ~80% confluence, the culture media were discarded, and freshly prepared media containing tested chemicals (the media were FBS-free for incubations within 12 h, but contained 10% FBS for incubations for 12 h or longer time) were supplemented. Cells were then incubated at 37 °C for indicated times. HL60 cells were similarly cultured in IMDM supplemented with 12% FBS and antibiotics. When reaching ~80% confluence, cells were harvested through centrifugation at 1200 rpm for 2 min. The culture media were discarded and cells were treated with freshly prepared media containing tested chemicals.

2.3. Cytotoxicity

Cytotoxicity was assessed with the MTT assay. Cells were exposed to tested chemicals at 37 °C for specified times. Then, the media were removed and a solution of 10 μl MTT (5 mg/ml) in 90 μl FBS-free DMEM per well was added. After incubation at 37 °C for 4 h, the media were discarded and 100 μl DMSO per well was added. The plates were shaken at ambient temperature for 3 min, and the optical density at 490 nm was recorded. In controls, cells were exposed to vehicle only. Viability was calculated relative to the controls. Six independent samples were used.

2.4. Comet assay

Standard comet assay (i.e., alkaline comet assay) was performed as described previously [34,38]. Briefly, after exposure to tested chemicals, L02 and HepG2 cells were subsequently harvested by trypsinization, and HL60 cells were harvested through centrifugation. Cells were washed once with D-Hank's solution and then resuspended in FBS-free media. Approximately 30,000 cells per sample were used, which were suspended in 30 μl media and mixed with 140 μl low melting point agarose (1%). Lysis of cells was performed in the lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10; 1% Triton X-100 and 10% DMSO were added fresh) at 4 °C for 1 h. 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. After neutralization with 400 mM Tris (pH 7.5) and staining with 40 μl PI (5 μg/ml), DNA was analyzed under a fluorescence microscope (Olympus BX-51, Japan).

To detect cross-links, a second genotoxic agent, MMS, was employed as recommended by the international expert panel for comet assay [31]. The second genotoxic agent generates additional strand breaks and cross-links induced by a chemical can be detected through retardation in the rate of DNA migration. For chemicals that can simultaneously induce both strand breaks and cross-links, it is critical that DNA damage caused by the second genotoxic agent must be severe enough, otherwise the retardation in the rate of DNA migration caused by the tested chemicals may be unable to be observed [34]. Like DEB, MBO is a bifunctional alkylating agent and can simultaneously cause strand breaks and cross-links. We already found, when insufficient concentrations of MMS were used, the rates of DNA migration kept increasing with the increase in the MBO concentration and thus MBO-induced cross-links failed to be observed. An experiment was thus carried out to search for an appropriate MMS concentration. Based on the result of this experiment, 500 μM MMS was selected, which could cause such severe DNA damage at 1 h that the rate of DNA migration reached 70–80%.

It has been demonstrated that adding the second genotoxic agent to cells before or after treatment with the cross-linking agent gave similar results [39]. Therefore, in the present study, cells were exposed to different concentrations of MBO (10, 50, 200, 500, and 1000 μM) first for specified times and then to 500 μM MMS for 1 h. The cells were then harvested and subjected to the standard comet assay as described above.

2.5. Statistical analyses

DNA percentage in the tail (%Tail DNA) was used as the metric for quantification of DNA migration based on its apparent advantages over other metrics [32,33,40–43]. 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 [44]. Each glass slide represented one independent sample. The intra-sample data in our experiments did not show normal distributions, as a result, medians, instead of arithmetic means, were extracted from the data and were subsequently used in the analyses of results as recommended by biostatisticians [43,45]. The values presented in the present study were the means and standard deviations (SD) of at least three independent samples. Student's *t*-test was used to examine the statistical significances of the differences between two groups of data. A *p*-value smaller than 0.05 was considered to indicate the statistical significance.

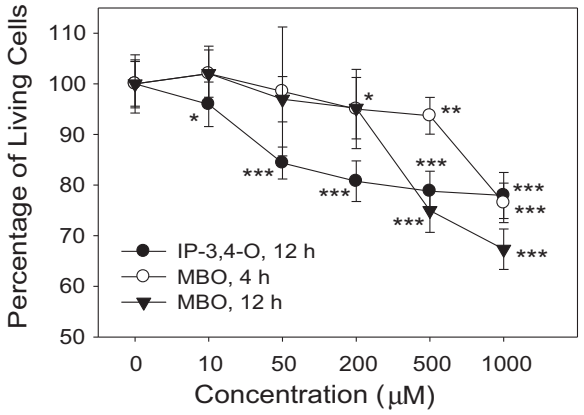

Fig. 2. Cytotoxicity of IP-3,4-O and MBO at different concentrations with different exposure times as measured by the MTT assay (IP-1,2-O did not exhibit cytotoxicity up to 1000 μM and 12 h, and thus its data were not shown) ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

3. Results

3.1. Cytotoxicity of IP-1,2-O, IP-3,4-O, and MBO

Because DNA damage can lead to cell death, it is critical that the highest dose tested in the comet assay does not induce excessive cytotoxicity. Therefore, cytotoxicity of IP-1,2-O, IP-3,4-O, and MBO was assessed with the MTT assay. Human hepatocyte L02 cells were exposed to the three chemicals at 10, 50, 200, 500, and 1000 μM for 1 h and no cytotoxicity was observed (data not shown). When cells were incubated with the chemicals for 4 h, only MBO exhibited slight cytotoxicity at high concentrations (500 and 1000 μM; the corresponding viability was 94% and 76%, respectively) (Fig. 2). With longer incubation time (12 h), IP-1,2-O still did not show any cytotoxicity, however, IP-3,4-O and MBO exhibited concentration-dependent cytotoxicity (Fig. 2). While IP-3,4-O exhibited statistically significant effects starting from 10 μM, MBO did so from 200 μM. At the highest concentration (1000 μM), the viability was 78% and 67% for IP-3,4-O and MBO, respectively.

3.2. Concentration-dependent genotoxicity profiles of IP-1,2-O, IP-3,4-O, and MBO as measured by the standard comet assay

To examine the genotoxic potential of IP-1,2-O, IP-3,4-O, and MBO at different concentrations, L02 cells were incubated with the chemicals at 10, 50, 200, 500, and 1000 μM for 1 h. The concentrations and incubation time were selected to facilitate comparison with the

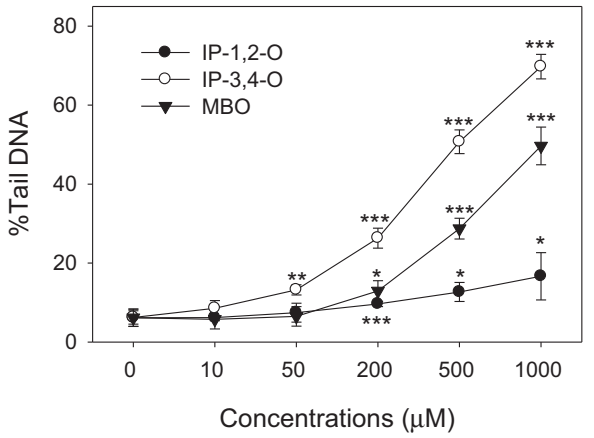

Fig. 3. The rates of DNA migration caused by three metabolites of isoprene (IP-1,2-O, IP-3,4-O, and MBO) at different concentrations with the incubation time being 1 h in human hepatocyte L02 cells as measured by the standard comet assay ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

results obtained previously using 1,3-butadiene metabolites [34,37,38]. The values for the controls were within the historical records in our laboratory.

As shown in Fig. 3, the three metabolites were all genotoxic. However, they exhibited rather different potency, especially at high concentrations (>200 μM). Among the metabolites, IP-1,2-O had the lowest potency; it caused statistically significant effects on the rates of DNA migration starting from 200 μM. At the highest concentration tested, the increase in the rate of DNA migration over the control was only moderate (16.7% vs. 6.2% of the control, $p < 0.05$). In contrast, IP-3,4-O exhibited the highest genotoxic potential; it was considerably more potent than IP-1,2-O at each concentration tested. At 10 μM, IP-3,4-O already induced an increase in the rate of DNA migration over the control, although the increase was not statistically significant ($p = 0.1$). Starting from 50 μM, the increases in the rates of DNA migration became statistically significant. At 1000 μM, IP-3,4-O caused a large increase in the rate of DNA migration over the control (69.8% vs. 6.2% of the control, $p < 0.001$). Actually, 1000 μM IP-3,4-O caused such great DNA damage that the rate of DNA migration has been close to the maximum (theoretically 100%, but practically 80–90%). Interestingly, the genotoxic potential of MBO was lower than that of IP-3,4-O. MBO caused statistically significant increases in the rates of DNA migration starting from 200 μM. At 1000 μM, MBO induced a rate of DNA migration at approximately 50% (Fig. 3). Therefore, the three metabolites showed a potency rank order of IP-3,4-O > MBO > IP-1,2-O.

Because the genotoxicity potential of IP-3,4-O was unexpectedly high, in particular, higher than MBO, the three metabolites were also tested in HepG2 and HL60 cell lines. Overall, IP-1,2-O, IP-3,4-O, and MBO all exhibited similar profiles to those observed in L02 cell line (Fig. 4). IP-1,2-O was still the weakest genotoxicant among the three chemicals. However, the relative potency of IP-3,4-O and MBO depended on the concentrations. At high concentrations (>200 μM), IP-3,4-O was still more potent than MBO; however, at low concentrations (≤200 μM), MBO

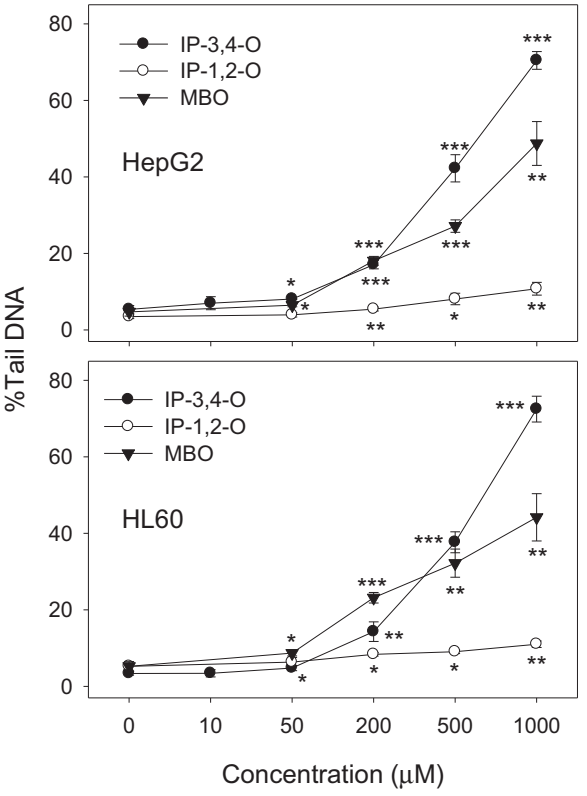

Fig. 4. The rates of DNA migration caused by IP-1,2-O, IP-3,4-O, and MBO at different concentrations with the incubation time being 1 h in HepG2 and HL60 cells as measured by the standard comet assay ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

showed the same potency as IP-3,4-O in HepG2 cells, and slightly higher potency than IP-3,4-O in HL60 cells (Fig. 4). Nonetheless, it was confirmed that IP-3,4-O still exhibited surprisingly high genotoxicity potential in HepG2 and HL60 cell lines, which was comparable or even higher than MBO.

3.3. Time-dependent genotoxicity profiles of IP-1,2-O, IP-3,4-O, and MBO

To examine how the rate of DNA migration caused by the metabolites changed with the incubation time, L02 cells were exposed to IP-1,2-O, IP-3,4-O, and MBO for 0.5, 1, 2, and 4 h. For IP-1,2-O, the highest concentration (1000 μM) was used. For IP-3,4-O, two lower concentrations (200 and 500 μM), rather than 1000 μM, were selected. The rate of DNA migration caused by 1000 μM IP-3,4-O has been close to the maximum (Fig. 3); as a result, there may not be sufficient room for further increases. For MBO, 200 and 1000 μM MBO were tested.

The results showed that the three metabolites exhibited different time-dependent profiles. For IP-1,2-O, the rate of DNA migration was the greatest at the first time point (0.5 h), and then decreased with the increase in the incubation time (Fig. 5). That is, IP-1,2-O showed a negative time-dependent genotoxicity profile. When cells were exposed to IP-1,2-O for 4 h, the observed rate of DNA migration was close to that in the control, although the

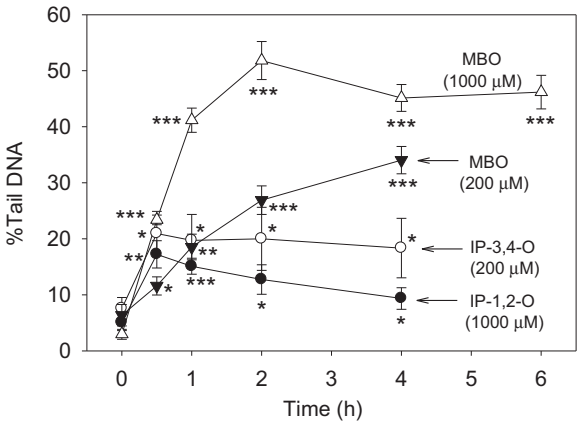

Fig. 5. The rates of DNA migration induced by 1000 μM IP-1,2-O, 200 μM IP-3,4-O, and 200 and 1000 μM MBO in human hepatocyte L02 cells with the incubation time being 0.5, 1, 2, and 4 h (an additional time point of 6 h was tested for 1000 μM MBO) as measured by the standard comet assay ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).

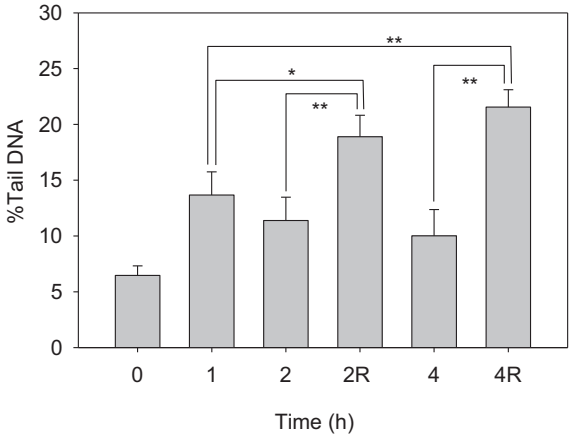

Fig. 6. The decrease in the rate of DNA migration caused by IP-1,2-O with the increase in the incubation time could be attributed to rapid hydrolysis of IP-1,2-O. Human hepatocyte L02 cells were incubated with 1000 μM IP-1,2-O for 1, 2, and 4 h. For comparison, cells were also incubated with 1000 μM IP-1,2-O for 2 and 4 h, but the IP-1,2-O-containing media were replaced with freshly prepared media every hour (which were indicated by 2R and 4R) ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$).

difference was still statistically significant. Longer incubation time led to a lower rate of DNA migration; when cells were incubated with 1000 μM IP-1,2-O for 12 h, the rate of DNA migration showed no statistically significant difference from that in the control (data not shown).

The situation was different for IP-3,4-O. The rate of DNA migration induced by 200 μM IP-3,4-O virtually did not change over the incubation time (Fig. 5). Although it seemed that the rate of DNA migration decreased very slightly with the increase in the incubation time, the decreases were not statistically significant at all. The result obtained with 500 μM IP-3,4-O was similar; the rate of DNA migration kept unchanged over the incubation time (data not shown).

MBO represented the third situation. Its time-dependent genotoxicity profile depended on the concentration. At 200 μM, MBO exhibited a positive profile, namely, the MBO-induced rate of DNA migration increased with the increase in the incubation time. However, 1000 μM MBO produced a mixed profile, which consisted of a positive one from 0.5 to 2 h and then a negative one from 2 to 4 h (Fig. 5). An increase in the incubation time from 2 h to 4 h led to a statistically significant decrease in the rate of DNA migration. To confirm the decrease, an additional time point (6 h) was examined. Indeed, the rate of DNA migration at 6 h was smaller than that at 2 h and the decrease was also statistically significant ($p < 0.05$), although the data at 6 h showed no statistically significant difference from that at 4 h (Fig. 5).

3.4. Hydrolysis of IP-1,2-O contributed to its negative time-dependent genotoxicity profile

It was somewhat unexpected that IP-1,2-O gave a negative time-dependent genotoxicity profile. Usually, one will expect a positive profile for a DNA-alkylating agent. After all, longer incubation time means that more sites on DNA are alkylated and thus more strand breaks are produced. We speculated that the negative profile of IP-1,2-O may be attributable to its rapid hydrolysis in solution, because the half-life of IP-1,2-O, which was reported as 1.25 h at pH 7.4 and 37 °C [23,24], was comparable to the time scales in our experiments. A simple calculation indicated that 76% of IP-1,2-O remained at 0.5 h, 57% did at 1 h, and only one third did at 2 h.

To examine the speculation, an experiment with the media in the cell cultures being replaced frequently using freshly prepared ones was performed. Specifically, for the L02 cells being exposed to IP-1,2-O for 2 and 4 h, the media, which contained 1000 μM IP-1,2-O, were replaced with freshly prepared ones every hour. The result was shown in Fig. 6. Indeed, as speculated, the L02 cells with the media being replaced every hour showed considerably greater rates of DNA migration than those treated regularly, and the differences were statistically significant (both $p < 0.01$; see Fig. 6, compare the data at 2 and 2R, and at 4 and 4R). Moreover, these cells also exhibited greater rates of DNA migration than those being incubated for 1 h, and the differences were statistically significant as well (see Fig. 6, compare the data at 1 and 2R ($p < 0.05$), and at 1 and 4R ($p < 0.01$)). That is to say, when cells were constantly exposed to freshly prepared media, the rate of DNA migration caused by IP-1,2-O increased with the increase in the exposure time. In other words, the time-dependent profile of IP-1,2-O was reversed from negative to positive in this situation. Thus, the results suggested that the negative profile of IP-1,2-O could be attributed to its rapid hydrolysis.

3.5. MBO induced DNA cross-linking at high concentrations and long incubation times

MBO at 1000 μM caused a decrease in the rate of DNA migration when the incubation time was increased from 2 h to 4 or 6 h (Fig. 5), suggesting that MBO induced DNA cross-linking, because the decreases could not be attributed to

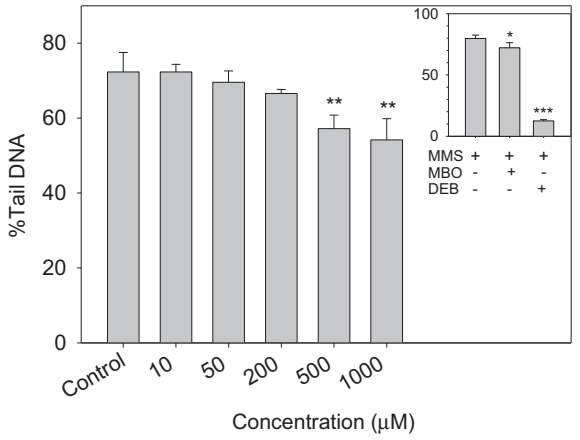

Fig. 7. Exposure to high concentrations of MBO for long time led to decreases in the rates of DNA migration caused by MMS in human hepatocyte L02 cells. Cells were exposed to different concentrations of MBO for 6 h and then 500 μM MMS for 1 h, and subsequently were subjected to the standard comet assay. Inset: Cells were incubated with 1000 μM MBO for 4 h and then 500 μM MMS for 1 h, and were subjected to the standard comet assay. Lower concentrations of MBO did not show any statistically significant effects, as a result, the data were not shown. As a positive control, cells were incubated with DEB (500 μM, 1 h) and then MMS ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$).

hydrolysis of MBO due to its long half-life (46 h) [23,24]. To confirm the presence of cross-links, L02 cells were incubated with different concentrations of MBO (10, 50, 200, 500, and 1000 μM) for 1, 2, 4, and 6 h, and subsequently 500 μM MMS for 1 h.

The results indicated that MBO failed to cause any retardation in the rate of MMS-induced DNA migration when cells were incubated with MBO for 1 or 2 h. However, under these conditions, the positive control (500 μM DEB) [34] led to a large decrease in the rate of DNA migration (which was statistically significant) (data not shown). Therefore, MBO did not induce detectable cross-links when the incubation time was 1 or 2 h. With the incubation time being 4 h, only 1000 μM MBO was observed to cause a small but statistically significant decrease (72.1% vs. 79.7% of the control, $p < 0.05$) in the rate of MMS-induced DNA migration (the inset in Fig. 7). When the incubation time was increased to 6 h, the effect became stronger; both 500 and 1000 μM MBO caused statistically significant decreases in the rates of MMS-induced DNA migration (57.2% and 54.2%, respectively, vs. 72.3% of the control, both $p < 0.01$). Therefore, the experiments using MMS not only confirmed the formation of MBO-induced cross-links, but also determined the conditions that detectable DNA cross-links were formed: 1000 μM MBO for 4 h, and 500 and 1000 μM MBO for 6 h.

4. Discussion

In the present study, we investigated the concentration- and time-dependent genotoxicity profiles of two monoepoxides and the diepoxide of isoprene in human hepatocyte L02 cells using the comet assay. The results indicated that the three metabolites all induced strand breaks in a concentration- and time-dependent manner, thus exhibiting genotoxicity. Interestingly, each metabolite showed unique features. Their genotoxic potency, which apparently depended on the concentration and incubation time, was quite distinct.

The concentration-dependent profiles of the three metabolites were all positive; however, their relative potency was rather different. In human hepatocyte L02 cells, IP-1,2-O and IP-3,4-O had the lowest and highest potential to induce strand breaks, respectively, with MBO lying in between. IP-3,4-O was more potent than both IP-1,2-O and MBO, especially at high concentrations (≥200 μM). In HepG2 cells, the above observation still stood, although virtually there was no difference between IP-3,4-O and MBO at low concentrations (≤200 μM). In HL60 cells, MBO was slightly more potent than IP-3,4-O at low concentrations (≤200 μM), however, IP-3,4-O still exhibited higher potential to induce strand breaks at high concentrations (>200 μM), especially at 1000 μM. Overall, among the three metabolites, IP-1,2-O was clearly the least potent in all three cell lines at all concentrations tested; at high concentrations (>200 μM), IP-3,4-O was undoubtedly the most potent in all three cell lines with MBO lying in between. At low concentrations (≤200 μM), depending on the cell line, the relative potency of IP-3,4-O and MBO varied.

It was beyond our expectation that IP-3,4-O had high potential to induce strand breaks. As it was unexpected, we first considered the possibility that the effect observed with IP-3,4-O was caused by some highly toxic impurities in the reagent. The purity of the reagent was 95.2% as determined by GC and the major impurity was dichloromethane (the residual solvent in the synthesis of IP-3,4-O, 1.5%). Its ^{1}H NMR spectrum indicated that the reagent had excellent purity and few peaks of impurities except dichloromethane were visible. Removal of dichloromethane by rotary evaporation, which was confirmed by the ^{1}H NMR spectrum, had no effect on the genotoxic potential of IP-3,4-O. Furthermore, we synthesized a small quantity of IP-3,4-O through the reaction of isoprene and mCPBA with a molar ratio of 1:1 in dichloromethane. This was a poor reaction because it was of low yield (the total yield of monoepoxides in our experiment was about 11%) and produced two monoepoxides with IP-1,2-O being the predominant product (the molar ratio of IP-1,2-O and IP-3,4-O in our synthesis was ~4:1, consistent with the literature [46]). The rationale behind the synthesis of IP-3,4-O using such a poor reaction was, if the reagent of IP-3,4-O did contain traces of highly genotoxic chemicals that were the residual reactants or were byproducts in the synthesis, the IP-3,4-O synthesized by ourselves would not contain these traces of chemicals because commercially IP-3,4-O would certainly not be synthesized using such a poor reaction (the commercial synthesis of IP-3,4-O probably uses the method reported by Harwood et al. [47]. It is a simple one-step reaction with a moderate yield). Because IP-1,2-O was the predominant product, in our synthesis, the IP-3,4-O, which was obtained by distillation, contained ~8% IP-1,2-O as indicated by the ^{1}H NMR spectrum. Despite the moderate purity, it produced a very similar result in L02 cells to IP-3,4-O obtained commercially. Therefore, the possibility that the strand breaks induced by the reagent of IP-3,4-O

were caused by impurities in the reagent could safely be excluded. That is to say, IP-3,4-O did have high potential in inducing strand breaks.

This was the most surprising finding in the present study. In fact, with the incubation time being 1 h, the genotoxic potential of IP-3,4-O in L02 cell line was not only the highest among the three metabolites of isoprene, but also exceeded that of monoepoxide and epoxydiol of 1,3-butadiene and 1-chloro-2-hydroxy-3-butene (an alternative metabolite of 1,3-butadiene), and was similar to that of DEB (however, only the data at 50 and 200 μM should be used to compare with DEB, because the rates of DEB-induced DNA migration at higher concentrations were smaller than that at 200 μM due to cross-linking) [34,37,38]. IP-3,4-O has low alkylating ability, which is only one tenth of MBO, and is thus non-mutagenic [24]. It has been demonstrated that mutagenicity of epoxides, as measured by the Ames test, was positively correlated with their alkylating ability [24]. This appears reasonable because the formation of DNA adducts is generally considered to be central to initiating the mutagenic process for DNA-reactive chemicals [48]. However, the formation of DNA adducts should also be the basis for genotoxicity of such chemicals. Therefore, it was surprising that IP-3,4-O had low alkylating ability and no mutagenicity but exhibited high genotoxicity. This might imply that other mechanisms are involved in the genotoxicity of IP-3,4-O. Bogaards et al. found that MBO could not explain the observed interspecies differences in the toxicity between mouse and rat, and thus assumed that other metabolites may play a role [49]. Because IP-1,2-O is easily hydrolyzed and thus has low toxicity, and the in vivo concentrations of MBO might be very low [5], it is possible that IP-3,4-O plays an important role in the toxicity of isoprene. Further studies should be conducted to address the issue.

Interestingly, the time-dependent profiles of the three metabolites exhibited trends different from each other. IP-1,2-O showed a negative profile, whereas MBO (200 μM) gave a positive one, with IP-3,4-O lying in between, that is, the rate of DNA migration did not change over the incubation time. In addition, 1000 μM MBO gave rise to a decrease in the rate of DNA migration for the incubation time of 4 h in comparison with that of 2 h.

To explain the time-dependent profile, it should be noted that the rates of DNA migration observed in the experiments actually represented the combined results of strand breaks and DNA repair. Alkylating agents induce strand breaks, which are repaired by the DNA repair machinery. The amounts of strand breaks induced by alkylating agents increase with time, therefore, how the rates of DNA migration observed in the experiments change with time is dependent upon the relative capability of alkylating agents and the DNA repair machinery. Strong alkylating agents cause rapid increases in the amounts of strand breaks over time, which overwhelm the capacity of the DNA repair machinery. As a result, the rates of DNA migration observed increase over time. MBO falls into this category, because it has high alkylating ability [24]. On the other hand, the amounts of strand breaks caused by weak alkylating agents also increase with time, however, if production of strand breaks is overwhelmed by the DNA repair machinery, the rates of DNA migration observed will decrease with time. This may explain the situation of IP-1,2-O. IP-1,2-O itself is a much weaker alkylating agent than MBO [24], and it is easy to be hydrolyzed. The two factors together lead to the negative time-dependent profile of IP-1,2-O. Indeed, the experiment replacing the IP-1,2-O-containing media demonstrated that its hydrolysis was the major contributor to the observed phenomenon. In addition, one can expect a third case, namely, when the amounts of production of strand breaks are more or less the same as those repaired by the DNA repair machinery, the rates of DNA migration observed will not change with time. It might be the case of IP-3,4-O.

However, obviously the negative profile of 1000 μM MBO from 2 to 4 h could not be explained by the above mechanism, because MBO is a strong alkylating agent, plus, a high concentration was used. This phenomenon was considered to be caused by the formation of cross-links, because MBO is a bifunctional alkylating agent. Indeed, the experiment with MMS confirmed the formation of cross-links at high MBO concentrations and long incubation time.

It was clear that the cross-linking potential of MBO was much weaker than that of DEB. MBO-induced cross-links were able to be detected only at high concentrations (500 and 1000 μM) and long incubation times (4 and 6 h), and the observed effects were not strong. Even under the best conditions (MBO at 1000 μM and the incubation time at 6 h), only a moderate decrease in the rate of DNA migration relative to the control was observed (54.2% vs. 72.3% of the control, $p < 0.05$). By contrast, DEB-induced cross-links were able to be easily detected at 500, 800, and 1000 μM with the incubation time being 1 h and the effects were strong; 1000 μM DEB reduced the rate of MMS-induced DNA migration from 63.7% of the control to 3.0% [34]. The results supported the proposition of Watson et al., who expected that MBO has lower cross-linking potential compared to DEB [2]. This was attributed to the methyl group in the molecule, which suppresses the nucleophilic attack to the oxirane ring carrying the methyl group through steric hindrance and instead enhances intramolecular cyclization or depurination [2].

In summary, in the present study we investigated the concentration- and time-dependent genotoxicity profiles of three isoprene metabolites and unexpectedly found that IP-3,4-O had surprisingly high potential to induce strand breaks. The underlying mechanisms deserve further studies.

Conflict of interest

The authors declare no conflict of interest.

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