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## Safrole-2',3'-oxide induces cytotoxic and genotoxic effects in HepG2 cells and in mice

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### ABSTRACT

Safrole-2',3'-oxide (SAFO) is a reactive electrophilic metabolite of the hepatocarcinogen safrole, the main component of sassafras oil. Safrole occurs naturally in a variety of spices and herbs, including the commonly used Chinese medicine Xi xin (*Asari Radix et Rhizoma*) and Dong quai (*Angelica sinensis*). SAFO is the most mutagenic metabolite of safrole tested in the Ames test. However, little or no data are available on the genotoxicity of SAFO in mammalian systems. In this study, we investigated the cytotoxicity and genotoxicity of SAFO in human HepG2 cells and male FVB mice. Using MTT assay, SAFO exhibited a dose- and time-dependent cytotoxic effect in HepG2 cells with TC<sub>50</sub> values of 361.9 μM and 193.2 μM after 24 and 48 h exposure, respectively. In addition, treatment with SAFO at doses of 125 μM and higher for 24 h in HepG2 cells resulted in a 5.1–79.6-fold increase in mean Comet tail moment by the alkaline Comet assay and a 2.6–7.8-fold increase in the frequency of micronucleated binucleated cells by the cytokinesis-block micronucleus assay. Furthermore, repeated intraperitoneal administration of SAFO (15, 30, 45, and 60 mg/kg) to mice every other day for a total of twelve doses caused a significant dose-dependent increase in mean Comet tail moment in peripheral blood leukocytes (13.3–43.4-fold) and in the frequency of micronucleated reticulocytes (1.5–5.8-fold). Repeated administration of SAFO (60 mg/kg) to mice caused liver lesions manifested as a rim of ballooning degeneration of hepatocytes immediately surrounding the central vein. Our data clearly demonstrate that SAFO significantly induced cytotoxicity, DNA strand breaks, micronuclei formation both in human cells *in vitro* and in mice. More studies are needed to explore the role SAFO plays in safrole-induced genotoxicity.

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### 1. Introduction

Safrole (4-allyl-1,2-methylenedioxybenzene, CAS Number: 00094-59-7), the main component of the essential oil in the root bark and the fruit of sassafras plants, occurs naturally in various amounts in numerous edible herbs and spices: e.g., basil, nutmeg, star anise, mace, cinnamon leaves [1]. Besides, safrole is found in the essential oil of commonly used Chinese medicine, such as Xi xin (*Asari Radix et Rhizoma*, up to 5300 ppm of dried herb) [2] and Dong

quai (*Angelica sinensis*, up to 40 ppm) [3]. In Taiwan, about 2 million people (10% of the population) chew betel quid, a mild stimulant comprising areca nut, slaked lime, and piper betel inflorescence [4]. High levels of safrole (15.4 mg/g wet weight) found in Piper betel inflorescence can lead to extremely high levels of safrole exposure (up to 420 μM) in saliva during betel quid chewing [4]. Safrole was banned by the United States Food and Drug Administration for use as flavorings and food additives in 1960 (Federal Register 1960, 25 FR 12412) because it caused hepatocarcinoma in rats when fed in the diet at doses of 390 and 1170 ppm for 2 years [5,6]. In 1976, the International Agency for Research on Cancer classified safrole as a Group 2B carcinogen (possible human carcinogen) [7].

*In vitro* mouse and rat hepatic microsomal studies and *in vivo* studies have shown that safrole is metabolized by the cytochrome P-450 pathway to an electrophilic epoxide metabolite, safrole-2'-3'-oxide (SAFO) [8–10]; a presumed proximate carcinogenic metabolite, 1'-hydroxysafrole [10–12]; and

Abbreviations: SAFO, safrole-2',3'-oxide; CBPI, cytokinesis-block proliferation index; MNRETs, micronucleated reticulocytes; RETs, reticulocytes.

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a reactive oxygen species (ROS)-forming metabolite, hydroxy-chavicol (1,2-dihydroxy-4-allylbenzene) [13,14]. A physiologically based biokinetic (PBBK) model for safrole in rats developed using *in vitro* metabolic parameters has shown that, at a dose of 300 mg/kg safrole, the percentage of safrole metabolized to 2',3'-dihydroxysafrole (derived from SAFO), 1'-hydroxysafrole, 3'-hydroxysafrole, and 1,2-dihydroxy-4-allylbenzene was 6.9%, 10.0%, 7.7%, and 74.0% of the dose, respectively [10]. The carcinogenic effects of SAFO and 1'-hydroxysafrole have been reported in mice [15,16], and 1'-hydroxysafrole is considered to be the main proximate carcinogenic metabolite of safrole [12]. SAFO can react directly with calf thymus DNA *in vitro* to produce at least eight SAFO-DNA adducts as measured by  $^{32}\text{P}$ -postlabeling analysis [17]. However, none of these DNA adducts were detected in liver tissues of male Balb/C mice treated with a single intraperitoneal dose (600  $\mu\text{mol/kg}$ ) of SAFO or safrole [17], probably because of the rapid metabolic inactivation of the compound by cytosolic and microsomal epoxide hydrolase and cytosolic glutathione S-transferase [18,19]. These data led to the assumption that the metabolism of safrole via epoxidation may not play a major role in the genotoxicity of safrole [17].

The contribution of SAFO to the genotoxicity of safrole still arouses interest because of its structural similarity to other known epoxide carcinogens like styrene oxide; its sufficient electrophilic reactivity to form DNA adducts *in vitro* [17,20]; its apparent formation in safrole-exposed rats, guinea pigs, and man, as evidenced by the detection of dihydrodiol metabolites in urine [8,21–23]; its considerable persistence in urine of SAFO-exposed rats and guinea pigs [8]; its pronounced mutagenicity in the Ames test [16,24], and its tumorigenicity in mice [15]. Miller et al. reported that CD-1 adult female mice given 24 topical applications (2 mg/application) of SAFO, followed by twice-weekly applications of croton oil, induced skin papillomas and keratoacanthomas in 36% of SAFO-treated mice, compared to 7% in control mice [15]. Moreover, SAFO was the most active metabolite of safrole tested in the Ames test, exerting dose-dependent mutagenic activities in *S. typhimurium* strains TA100 [16] and TA1535 [16,24] in the absence of metabolic activation, with specific mutagenic activities of 6000 and 6000–7100 revertants/ $\mu\text{mol}$ , respectively. Although SAFO has been shown to be efficiently detoxified, SAFO was detected not only in urine of rats and guinea pigs treated with SAFO [8], but also in urine of rats treated with safrole [9]. The above evidences suggest that some of SAFO can escape detoxification that in turn has the potential to cause DNA damage *in vivo*.

Currently, there is little or no published data on the mutagenicity and genotoxicity of SAFO in mammalian systems either *in vitro* or *in vivo*. This study investigated the cytotoxicity and genotoxic effects of SAFO by MTT, alkaline Comet, and micronucleus assays in HepG2 cells and by the histopathological microscopy in liver, alkaline Comet, and micronucleus assays in peripheral blood cells of FVB mice to better understand the role that SAFO plays in the genotoxicity of safrole. In HepG2 cells, SAFO induced cytotoxicity in a dose- and time-dependent manner, and resulted in a dose-dependent increase in mean Comet tail moment and the frequency of micronucleated binucleated cells. In male FVB mice, repeated administration of SAFO caused liver damage, typically manifested as a rim of ballooning degeneration of hepatocytes immediately adjacent to the central vein. SAFO caused a significant dose-dependent increase in mean Comet tail moment of

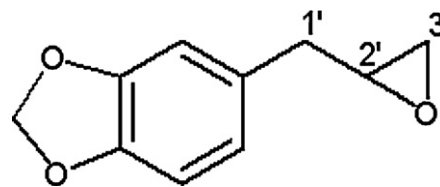


Fig. 1. Chemical structure of safrole 2',3'-oxide (SAFO).

cytotoxic and genotoxic effects both in human cells *in vitro* and in mice.

## 2. Materials and methods

### 2.1. Chemicals

Fetal bovine serum was obtained from HyClone (Logan, Utah, USA). Dulbecco's Modified Eagle's Medium (DMEM), penicillin, and streptomycin were purchased from Life Technologies (Gaithersburg, Maryland, USA). Acridine orange (AO), dimethyl sulfoxide (DMSO), propidium iodide (PI), MTT, agarose (normal and low melting point), and cytochalasin B were purchased from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade and were also obtained from Sigma.

### 2.2. Preparation of SAFO

SAFO was synthesized following previously published procedures [25]. Briefly, *m*-chloroperbenzoic acid (30 g, 0.17 mol) in 200 mL chloroform was slowly added to a solution of safrole (22.7 mL, 0.15 mol) in 50 mL chloroform at 0 °C and was stirred at room temperature overnight. The reaction was terminated with 10% sodium sulfite. The reaction product was extracted 3 times with 250 mL of 5%  $\text{NaHCO}_3$  and 2 times with 200 mL of water. The organic layers were combined and evaporated to dryness. The residue was subjected to column chromatography with neutral silica gel and eluted with hexane/EtOAc (10:1), resulting in the final product as a light yellow liquid. The yield was 39.7%. The product was further characterized and confirmed as SAFO by liquid chromatography tandem mass spectrometry (LC-MS/MS) and nuclear magnetic resonance (NMR) spectroscopy. ESI+/MS:  $m/z$  179 ( $[\text{M}+\text{H}]^+$ );  $^1\text{H}$  NMR (300 MHz, in  $\text{CDCl}_3$ ):  $\delta$  2.51 (dd, 1H, H- $\gamma'$ ,  $J=2.6$  Hz,  $J=4.9$  Hz), 2.70–2.81 (m, 3H, H- $\gamma''$ , H $\alpha'$ , H $\alpha''$ ), 3.06–3.11 (m, 1H, H- $\beta$ ), 5.91 (s, 2H, CH<sub>2</sub>), 6.66–6.69 (m, 1H, Ar-CH), 6.73–6.75 (m, 2H, Ar-CH).  $^{13}\text{C}$  NMR (75.4 MHz, in  $\text{CDCl}_3$ ):  $\delta$  46.7 (C- $\alpha$ ), 52.5 (C- $\beta$ ), 38.3 (C- $\gamma$ ), 108.2 (Ar-CH), 130.7 (Cq), 122.8 (Ar-CH), 109.4 (Ar-CH), 146.2 (Cq), 147.6 (Cq), 100.8 (C-g). The purity of the synthesized SAFO was estimated by HPLC-UV chromatography to be 96.5% (the ratio of its peak area to the total area of all peaks). The chemical structure of SAFO is shown in Fig. 1.

### 2.3. Cell culture and exposure

A human hepatoma HepG2 cell line was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100  $\mu\text{g/ml}$  streptomycin in 75 cm<sup>2</sup> tissue culture flasks in a humidified incubator at 37 °C under an atmosphere containing 5%  $\text{CO}_2$ . Cells in the logarithmic growth phase were treated with different concentrations of SAFO (0, 125, 250, 312.5 and 375  $\mu\text{M}$ ) for 24 or 48 h before MTT analysis and for 24 h before the alkaline Comet assay and micronucleus test were performed.

### 2.4. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay

Cell viability was determined by a MTT colorimetric assay. MTT was purchased from Sigma and dissolved in phosphate-buffered saline (137 mM NaCl, 1.4 mM  $\text{KH}_2\text{PO}_4$ , 4.3 mM  $\text{Na}_2\text{HPO}_4$ , 2.7 mM KCl, pH 7.2). Briefly, cells were seeded in 96-well culture plates and incubated overnight before being treated with different concentrations of SAFO (0, 125, 250, and 375  $\mu\text{M}$ ) dissolved in DMSO. The maximum DMSO concentration in culture was 0.1% (v/v). After a 24-h incubation period, a one-tenth volume of 5 mg/ml MTT was added to the culture medium, and incubated for 4 h in the dark. An equal volume of solubilization solution (10% SDS/0.1 N HCl) was then added, and the absorbance was measured at a wavelength of 570 nm and 650 nm by a SpectraMax<sup>®</sup> 340PC384 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA). The relative survival rate was calculated using the following equation:

$$\text{Relative cell survival rate (\%)} = \frac{(\text{Absorbance of SAFO-treated cells} - \text{Absorbance of medium only})}{(\text{Absorbance of DMSO-treated cells} - \text{Absorbance of medium only})} \times 100\%$$

peripheral blood leukocytes and in the frequency of micronucleated reticulocytes. Our data demonstrate that SAFO exhibits significant

The  $\text{TC}_{50}$  values (50% toxic concentration) were determined as the concentration of SAFO required to reduce cell viability by 50% relative to untreated control cells. Values are reported as mean  $\pm$  standard deviation of three independent experiments.

### 2.5. Comet assay (alkaline single-cell gel electrophoresis) in HepG2 cells [26]

After 24 h exposure to SAFO, the alkaline version of the Comet assay was performed. A 10  $\mu$ l aliquot of cell suspension ( $1 \times 10^4$  cells) was gently mixed with 60  $\mu$ l of 0.5% normal-melting agarose in PBS at 37 °C. Then, 65  $\mu$ l of this cell suspension in normal-melting agarose was placed on a microscope slide that had been pre-coated with 75  $\mu$ l of 1% solidified low-melting agarose. A coverslip was placed on the cell suspension in agarose and was allowed to set for 10 min on ice. After the agarose had solidified, the coverslip was removed and the slide was immersed in a lysis buffer (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Tris, 1% Triton X-100, and 10% DMSO, pH = 10) at 4 °C for 1 h. After lysis, the slides were placed in a horizontal gel electrophoresis tank containing a cold freshly prepared alkaline buffer (300 mM NaOH and 1 mM Na<sub>2</sub>EDTA, pH > 13) for 20 min to allow DNA to unwind. Thereafter, electrophoresis was performed at 25 V (1.15 V/cm), which was adjusted by raising or lowering the buffer level in the tank for 20 min. After electrophoresis, the slides were immediately neutralized with 0.4 M Tris buffer (pH = 7.5) at 4 °C for 15 min, fixed with methanol for 5 min, and allowed to dry at room temperature. Finally, the slides were stained with propidium iodide (5  $\mu$ g/ml) and observed under an inverted fluorescence microscope (Olympus CKX41 and U-RFLT 50) (Olympus Co. Ltd., Tokyo, Japan) with a 20 objective. Comet images were captured by a digital charge-coupled device (CCD) camera (Olympus DP70, Olympus Co. Ltd., Tokyo, Japan) under fluorescence microscope.

The extent of DNA damage was analyzed in 50 randomly selected cells (25 cells from each of two replicate slides) from each sample using TriTek CometScore™ version 1.5 software (TriTek Corp., Sumerduck, VA, USA). The basic assumption is that the amount of DNA at a location is proportional to the pixel intensity at that position. Tail moment is defined in arbitrary units as the % DNA in tail multiplied by the tail length, divided by 100. All slides were coded and examined in a double-blind manner to avoid observer bias. Some Comet data were not normally distributed, and the median differed substantially from the mean. Therefore, the median values of the tail moment for each experimental sample were calculated. Values are reported as mean  $\pm$  standard deviation of four replicates from two independent experiments.

### 2.6. Cytokinesis-block micronucleus test in HepG2 cells

HepG2 cells were seeded on coverslips overnight and then treated with different concentrations of SAFO (0, 125, 250, and 312.5  $\mu$ M) for 24 h. SAFO at 375  $\mu$ M was too toxic to be evaluated. After a 24 h incubation period, cytochalasin B was added to reach a final concentration of 3  $\mu$ g/ml and incubated for another 16 h to obtain binucleated cells. Then, the cells on coverslips were fixed with methanol at 4 °C for 30 min, air-dried for 10 min, and stained with acridine orange (0.2 mg/ml) in the dark for 10 min. Finally, the slides were examined under a fluorescence microscope at an excitation wavelength of 488 nm. For each treatment, the frequency of micronuclei formation was scored in 1000 binucleated cells. Cytokinesis-block proliferation index (CBPI) was used as a parameter for cytotoxicity and was calculated by screening 500 cells per treatment group for the frequency of cells with one or more nuclei using the following formula.

$$CBPI = \frac{M1 + 2(M2) + 3(M3 + M4)}{N}$$

where M1–M4 is the number of cells with 1–4 nuclei and *N* is the total number of cells scored [27]. All slides were coded and examined in a double-blind manner to avoid observer bias. Values are reported as mean  $\pm$  standard deviation of four replicates from two independent experiments.

### 2.7. Animals and dosing

Mouse experiments were conducted in accordance with ethics approval from China Medical University Animal Ethics Committee. Male FVB mice, 6–7 weeks old weighing 20–25 g, were purchased from the National Laboratory Animal Center (Taipei, Taiwan). Animals were acclimatized for about seven days prior to SAFO exposure. The mice were divided into 5 groups of 4 animals each. SAFO was dissolved in olive oil, and the dosing volume of SAFO in olive oil was 3.3 ml/kg body weight. SAFO was administered by intraperitoneal injection at doses of 15, 30, 45, and 60 mg/kg every other day for 24 consecutive days. Controls received equal volume (3.3 ml/kg) injections of olive oil. All animals were allowed free access to food and water during the experiment. In addition, all mice were kept under observation and weighed during the experiment.

### 2.8. Histopathological examination

Mice were sacrificed by CO<sub>2</sub> asphyxiation six days after the last administration. Blood samples were collected by intracardiac puncture into heparin-containing blood collection tubes, and then centrifuged at 3000 rpm for 10 min at 4 °C. Plasma levels of glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), plasma creatinine, and plasma blood urea nitrogen were measured with an autoanalyzer. At necropsy, a gross pathological examination was performed. The

liver and lung were dissected and their absolute and relative weights were evaluated. The liver specimens were then fixed in buffered formalin and stained with hematoxylin and eosin for histological examination. All liver biopsy sections were processed for light microscopy examination (HE stain). Histopathological evaluation was performed blinded by an experienced pathologist from the Department of Pathology, Chung Shan Medical University Hospital, Taichung, Taiwan.

### 2.9. Comet assay in mouse peripheral blood leukocytes

At 16 h after the last administration, the peripheral blood (10  $\mu$ l) was collected from a tail vein using a heparin-coated micropipette tip. The alkaline Comet assay was then performed as described above for HepG2 cells.

### 2.10. Micronucleus test with mouse peripheral blood reticulocytes

A 10  $\mu$ l volume of aqueous acridine orange (AO) solution (0.2 mg/ml) was spread homogeneously on a glass slide that had been pre-heated to about 70 °C and allowed to dry at room temperature. The prepared slides were stored in a dark and dry location at room temperature for at least 4 h before use. At 24 h after the last administration, about 10  $\mu$ l of peripheral blood was collected from a tail vein through a small cut made with a sharp-pointed scissor, immediately dropped onto the center of AO-coated slides, and then covered with a clean coverslip. The slides were subsequently kept at 4 °C in the dark for at least 4 h and examined under a fluorescence microscope at an excitation wavelength of 488 nm. At least two slides were examined for micronuclei formation per animal. The frequency of micronucleated reticulocytes (MNRETS) was recorded based on the observation of 1000 reticulocytes (RETS) per slide. All slides were coded for a “double-blind” analysis.

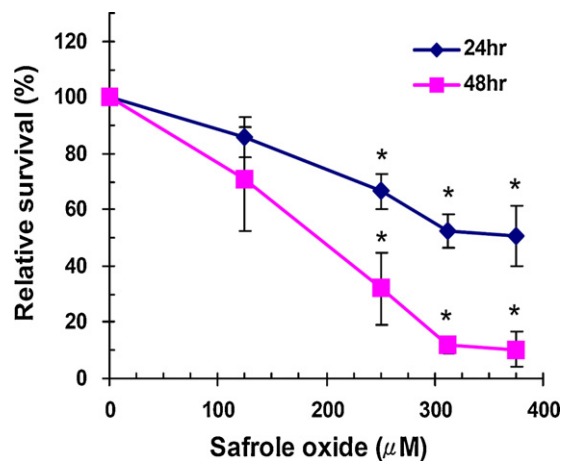
### 2.11. Statistical analysis

The data were expressed as a mean  $\pm$  standard deviation, and were tested for normality using the Shapiro–Wilk test and the Kolmogorov–Smirnov test using the Statistical Analysis Software (SAS) package (Version 9.1, SAS Institute, Cary, NC). With some data from MTT, Comet, or MN assays showing non-normal distribution ( $P < 0.05$ ), all two-group comparisons were performed with the nonparametric Wilcoxon rank-sum test as provided in PROC NPAR1WAY of SAS software package Version 9.1. The level for statistical significance was set at  $P < 0.05$  [28].

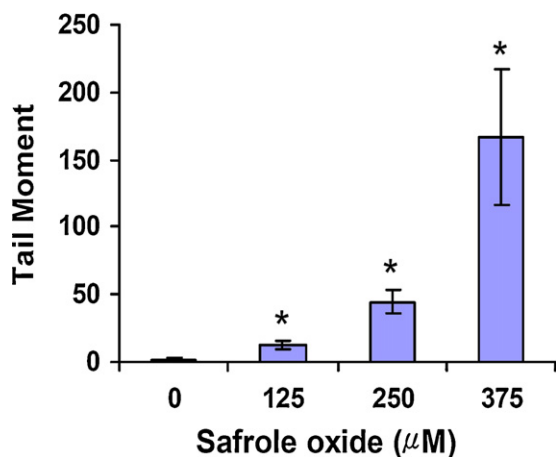
## 3. Results

### 3.1. Cytotoxicity in HepG2 cells

The viability of HepG2 cells exposed to SAFO at the doses of 125, 250, 312.5, and 375  $\mu$ M for 24 or 48 h was examined using a MTT assay. SAFO produced toxicity in HepG2 cells in a dose- and time-dependent manner (Fig. 2). Based on the survival curves, the SAFO concentrations required to achieve 50% cell survival after 24 or 48 h exposure were estimated to be approximately 361.9  $\mu$ M and 193.2  $\mu$ M, respectively.



**Fig. 2.** SAFO induced cytotoxicity in HepG2 cells as measured by MTT assay. HepG2 cells were treated with different concentrations of SAFO for 24 or 48 h. The medium was then replaced with fresh medium containing 0.5 mg/ml MTT. The absorbance at 570 and 650 nm of test and control wells was read to calculate the relative survival rate. Values are reported as mean  $\pm$  standard deviation of three independent experiments. \* $p < 0.05$  as compared to control cells.



**Fig. 3.** SAFO induces DNA strand break in HepG2 cells as measured by Comet assay. HepG2 cells were treated with different concentrations of SAFO for 24 h. Cells were embedded in agarose, subjected to the alkaline Comet assay, and stained with propidium iodide. The mean Comet tail moment from 50 cells in each treatment was calculated using TriTek CometScore™ version 1.5 software. Values are reported as mean  $\pm$  standard deviation of four replicates from two independent experiments. \* $p < 0.05$  as compared to control cells.

### 3.2. Comet assay in HepG2 cells

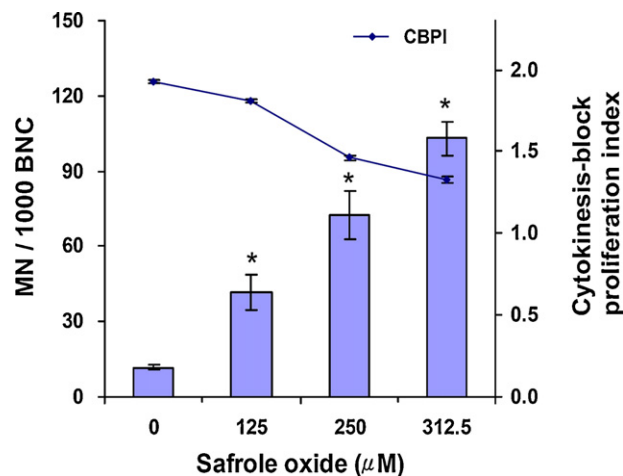
The degree of SAFO-induced DNA damage in HepG2 cells after 24 h exposure was analyzed by the alkaline Comet assay, which can detect DNA single strand breaks, alkali-labile apyrimidinic/apurinic sites, and transient repair sites. The Comet tail moment in SAFO-treated cells was quantified using CometScore software and then summarized as the median of 50 cells. Compared with background DNA damage obtained in control cells, SAFO resulted in a significant dose-dependent increase in the degree of DNA damage at concentrations of 125  $\mu\text{M}$  and above (Fig. 3). Exposure to SAFO at doses of 125  $\mu\text{M}$  and higher resulted in a 5.1–79.6-fold increase in mean Comet tail moment.

### 3.3. Cytokinesis-block micronucleus test in HepG2 cells

The cytotoxic and genotoxic effects of SAFO were further evaluated using the cytokinesis-block micronucleus test. In control cells, the percentage of binucleated cells was  $>75\%$  and the CBPI values were around 1.9. The background frequency of binucleated micronucleated cells ranged from 1.0% to 1.3%, which is within the normal range as reported in the literature or within our laboratory's historical data. SAFO treatment decreased the percentage of binucleated cells in a dose-response manner. In SAFO-treated cells, mean CBPI values observed at doses above 125  $\mu\text{M}$  were significantly lower than those in the negative control cells ( $P < 0.05$ ), with CBPI values of 1.3 at 312.5  $\mu\text{M}$  (Fig. 4). There was a significant dose-dependent increase in the frequency of binucleated micronucleated cells. The frequencies of micronucleated binucleated cells were significantly increased 2.6-, 5.2-, and 7.8-fold at 125, 250, and 312.5  $\mu\text{M}$ , respectively.

### 3.4. General toxicity evaluation in mice

Furthermore, we investigated whether SAFO has a significant cytotoxic or genotoxic effect *in vivo*. FVB mice were subjected to intraperitoneal injections of SAFO (15, 30, 45, and 60 mg/kg) every other day for 24 consecutive days, and then euthanized by  $\text{CO}_2$  six days after the last treatment. During the experimental period, all mice survived with the exception of one mouse in the group that received 60 mg/kg SAFO after the first treatment. This mouse was probably died from accidental death because all mice in a



**Fig. 4.** SAFO induced chromosome damage in HepG2 cells as measured by cytokinesis-block micronucleus test. HepG2 cells were treated with different concentrations of SAFO for 24 h. Following treatment, cells were exposed to cytochalasin B (3  $\mu\text{g}/\text{ml}$ ) for 16 h to obtain binucleated cells, and then cells were fixed and stained with acridine orange (0.2 mg/ml). The number of micronucleated cells was scored under fluorescent microscopy. A total of 1000 intact interphase cells were scored for each treatment. Values are reported as mean  $\pm$  standard deviation of four replicates from two independent experiments. BNC: binucleated cells; CBPI: cytokinesis-block proliferation index. \* $p < 0.05$  as compared to control cells.

follow-up study were survived after i.p. administration of 12 doses of 60, 90, or 120 mg/kg SAFO.

The behavior, coat color, and food consumption remained normal throughout the experiment. In addition, there were no significant differences in changes in body weight (Data not shown). At necropsy, no gross abnormalities were found.

### 3.5. Blood chemistry and histopathology

There were no significant differences in the relative weights (percentage of body weight) of liver between the experimental and control groups. We also observed no significant differences in GOT, GPT, BUN, or creatinine levels in serum between the experimental and control groups (data not shown). Histopathological examination showed normal architecture in the liver tissues of the control group. There were no significant morphological alterations in liver sections from mice treated with SAFO at the doses 15, 30 or 45 mg/kg every other day for 24 days. Furthermore, in the highest dose group (60 mg/kg), SAFO caused prominent ballooning degeneration and cytoplasmic vacuolation in the single layer of hepatocytes immediately surrounding the central vein, whereas, the hepatic cells around the portal vein area remained intact (Fig. 5).

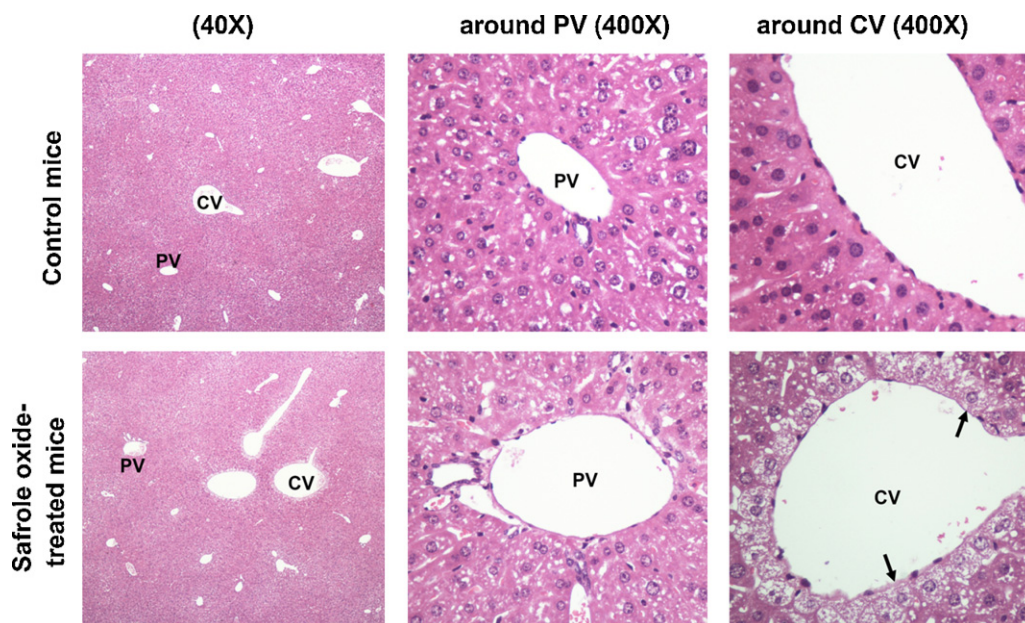
### 3.6. Comet assay in mouse peripheral blood leukocytes

At 16 h after the last SAFO administration, 10  $\mu\text{l}$  of peripheral blood was collected from each animal and subjected to the alkaline Comet assay. As shown in Fig. 6, SAFO at all doses resulted in a significant increase in the mean Comet tail moment relative to the control ( $P < 0.05$ ). Exposure to SAFO at doses of 15 mg/kg and higher resulted in a 13.3–43.4-fold increase in mean Comet tail moment.

### 3.7. Micronucleus test with mouse peripheral blood reticulocytes

We also evaluated the frequency of micronuclei in FVB mice. The frequency of the micronucleated reticulocytes provides an index of cytogenetic damage in mice. SAFO treatment resulted in a significant dose-dependent increase in the number of MNRETs per 1000





**Fig. 5.** Pathological changes in mouse liver after twelve doses of SAFO (60 mg/kg). Representative photomicrographs of HE staining of liver sections from control mice and SAFO-treated mice are shown at two magnifications (40 $\times$  and 400 $\times$ ). In SAFO-treated mouse liver, vacuolation and ballooning degeneration of hepatocytes immediately adjacent to the central vein are manifested. CV: central vein, PV: portal vein. Vacuolization of hepatocytes (arrows).

RETs (Fig. 7). The frequency of MNRETs increased 1.5-, 2.6-, 4- and 5.8-fold after administration of 15, 30, 45 and 60 mg/kg of SAFO, respectively.

#### 4. Discussion

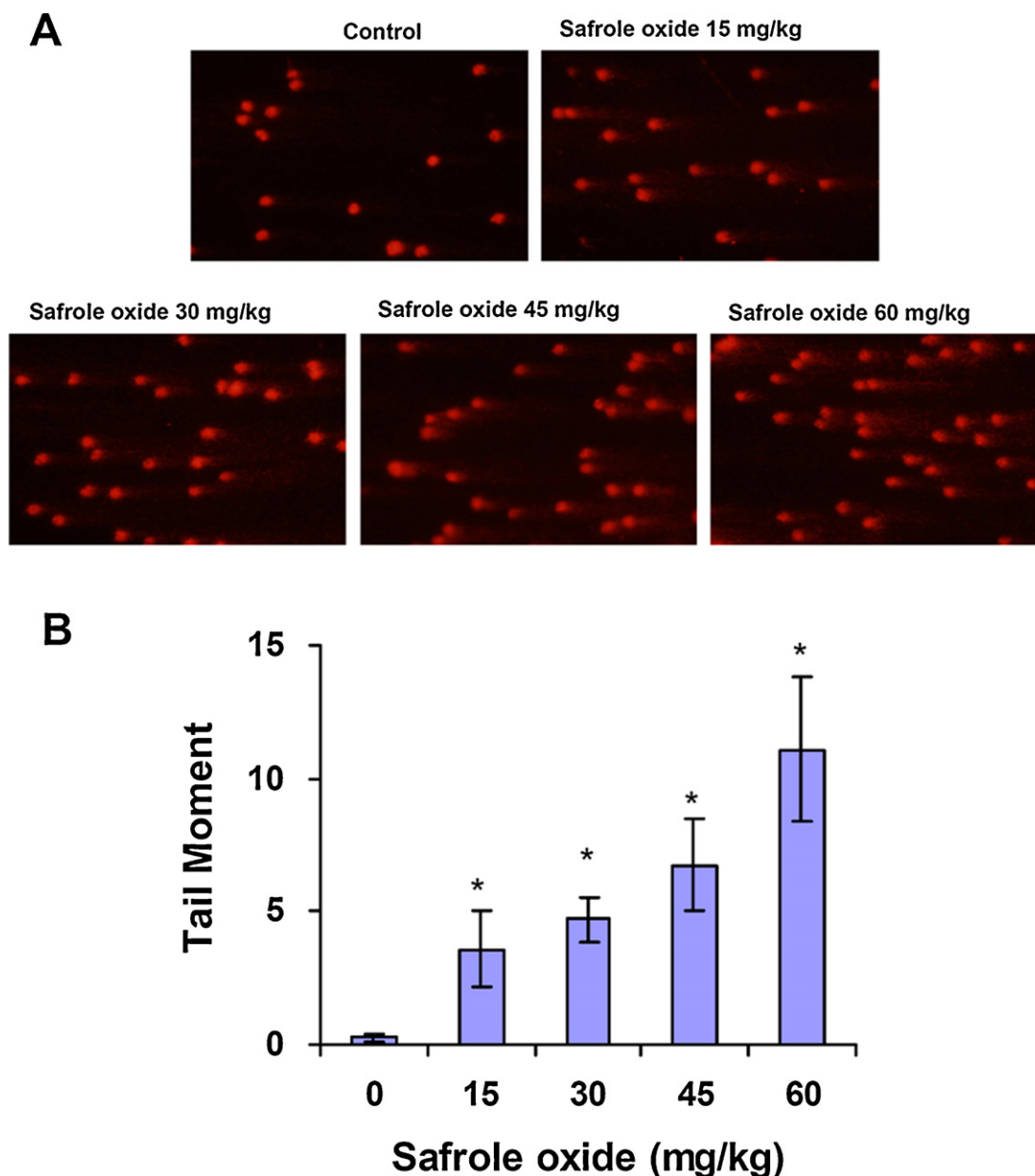
SAFO, a reactive epoxide metabolite of safrole, has been shown to induce DNA adduct formation *in vitro* [17,20], to be mutagenic in the Ames test in the absence of metabolic activation [16,24], and to cause tumors in mice [15]. Many epoxides are very reactive electrophiles that react readily with cellular DNA and form covalently bound DNA adducts [29]. Since the above experimental observations consistently point to direct DNA-reactivity of SAFO, we hypothesized that SAFO would be genotoxic in mammalian systems. In this study, we showed clear evidence that SAFO induced pronounced cytotoxicity and genotoxicity in human cultured cells and in mice.

Safrole has been shown to cause liver cancer and DNA adduct formation in rodents [5,6,30]; however, negative mutagenic and genotoxic results have been reported even in the presence of metabolizing rat liver homogenate fraction (S9 mix) in several biological test systems *in vitro*, such as in the Ames test [16,24,31], the Chinese hamster V79/*hprt* gene mutation assay [32], the Chinese hamster V79/ $\text{Na}^+/\text{K}^+$  ATPase gene mutation assay [33], and the mouse lymphoma L5178Y/*tk* assay [34]. The negative findings are presumed to be a result of inadequate metabolic activation in the test systems. Thus, the above data imply that adequate metabolic systems are required when the genotoxicity and mutagenicity of safrole or its metabolites are investigated. The metabolically competent human hepatoma HepG2 cell line retains activities of various Phase I and Phase II enzymes that are involved in the activation or detoxification of environmental genotoxicants. That cell line, therefore, better represents the biotransformation of these compounds *in vivo* than metabolically incompetent cell lines in the presence of exogenous metabolic activation [35]. Hence, in this study, we chose HepG2 cells as an *in vitro* model system to examine the genotoxic effects of SAFO.

Previous work with HepG2 cells showed that safrole induced the formation of DNA adducts [36], Comet tails [37], micronuclei,

and sister chromatid exchanges [38]. Uhl et al. found that exposure of HepG2 cells to safrole at a concentration of 4500  $\mu\text{M}$  for 24 h resulted in a 3.0-fold increase in Comet tail length relative to the control [37]. We found that treatment with 125  $\mu\text{M}$  SAFO for 24 h led to a 16.7-fold increase in Comet tail length in HepG2 cells (Fig. 3). HepG2 cells have relatively normal expression of most Phase II enzymes, but have lower expression of cytochrome P450 enzymes relative to those in normal human liver [39–41]. That may partly explain the observation that treatment of HepG2 cells with safrole led to a significantly positive result in the Comet assay only at concentrations of safrole equal to or higher than 4500  $\mu\text{M}$  [37]. Natarajan and Darroudi observed that the frequency of micronucleated binucleated cells increased by about 2.2-fold in HepG2 cells that had been treated for 28 h with 300  $\mu\text{M}$  safrole [38]. By comparison, in this study, treatment with 312.5  $\mu\text{M}$  SAFO for 24 h resulted in a 7.8-fold increase in the frequency of micronucleated binucleated HepG2 cells (Fig. 4). These results indicate that SAFO is more genotoxic than its parent compound, and thus may partly play a role in the genotoxicity of safrole.

Many epoxides are detoxified by epoxide hydrolases and glutathione S-transferases [18,19,29]. The lack of SAFO-DNA adducts in mice given safrole and SAFO are generally thought to occur as a result of the rapid and efficient metabolic inactivation of SAFO [18,19]. Safrole is an alkoxy derivative of allylbenzene. Guenther et al. compared the abilities of liver homogenates from several species of mammals, including guinea pig, rat, mouse, rabbit, and human, to detoxify allylbenzene 2',3'-oxide and found that human livers had the highest allylic epoxide hydrolase activity among the species tested [19]. The epoxide hydrolase activity of frozen human liver was about seven to ten times higher than that observed in mouse and rat liver [19]. In addition, kinetic studies showed that the activity of microsomal and cytosolic epoxide hydrolases in mouse liver with SAFO as the substrate was similar to that of allylbenzene 2',3'-oxide, in terms of  $K_m$  and  $V_{max}$  [18,19]. These data imply that human liver may provide greater protection against SAFO-induced cytotoxicity and genotoxicity than mouse or rat liver. In contrast, human glutathione S-transferase activities toward allylbenzene oxide are only two to four times lower than those measured in mouse and rat liver [19]. Although Phase II enzyme activity

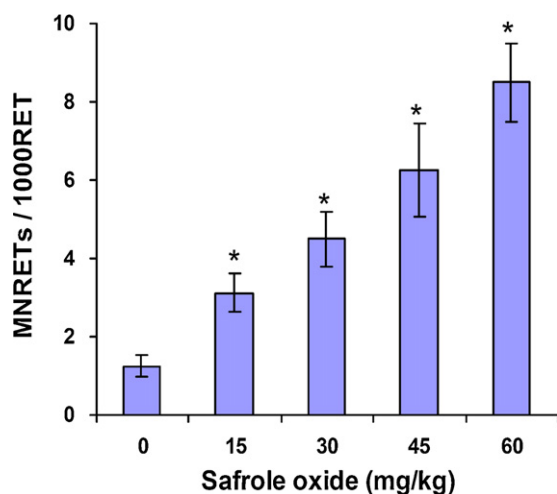


**Fig. 6.** SAFO induces DNA strand break in mouse peripheral blood leukocytes. SAFO was administered to mice by intraperitoneal injection at doses of 15, 30, 45 and 60 mg/kg every other day for 24 days. Peripheral blood collected from a tail vein at 16 h after the last administration was embedded in agarose, subjected to the alkaline Comet assay, and stained with propidium iodide. Mean Comet tail moment was scored in 100 cells in each animal. Values are indicated as mean  $\pm$  standard deviation, \* $p < 0.05$  as compared to control mice ( $n = 4$ ).

in HepG2 cells is similar to that in primary human hepatocytes [40,41], we still observed that SAFO significantly induced micronucleus formation in binucleated cells and extended the tail length even at the lowest dose tested (125  $\mu$ M) (Fig. 4). Therefore, even though SAFO was reported to be rapidly and efficiently detoxified by microsomal epoxide hydrolase and glutathione S-transferase [18,19], the marked genotoxic effects of SAFO observed in this study (Figs. 4 and 5) suggest that SAFO may be a highly reactive molecule capable of causing DNA damage and cytogenetic changes before the detoxification reactions occur. Further studies are needed to delineate the role SAFO plays in safrole-induced genotoxicity and carcinogenicity.

Daimon et al. found that safrole was genotoxic *in vivo* [30]. In their study, treatment of F344 rats with five doses of either 62.5 and 125, or 125 and 250 mg/kg safrole resulted in significantly increased levels of safrole-DNA adducts and increased

frequencies of sister chromatid exchanges and chromosome aberrations in the hepatocytes of rats [30]. Mughal et al. demonstrated a positive correlation between the induction of micronuclei frequency in the peripheral blood erythrocytes and different parameters from Comet assay in the peripheral blood lymphocytes of juvenile rats [42]. Using these techniques, we observed a significant dose-dependent increase in mean Comet tail moment in peripheral blood leukocytes (13.3–43.4-fold) and in the frequency of micronucleated reticulocytes (1.5–5.8-fold) after repeated intraperitoneal administration of SAFO (15, 30, 45, and 60 mg/kg) to mice. In contrast to our positive findings on the genotoxicity of SAFO, the only previous study that attempted to detect SAFO-DNA adducts *in vivo* by  $^{32}$ P-postlabeling analysis failed to detect these adducts in liver tissue isolated 24 h after intraperitoneal injection of a single dose of SAFO (106.9 mg/kg) or safrole (97.3 mg/kg) to male Balb/C mice [17]. Further studies that use liquid



**Fig. 7.** SAFO induced MNRETs in mouse peripheral blood. SAFO was administered to mice by intraperitoneal injection at doses of 15, 30, 45 and 60 mg/kg every other day for 24 days. Peripheral blood was collected from a tail vein at 24 h after the last administration, and then analyzed using a mouse micronucleus test. The number of micronucleated reticulocytes was recorded based on the observation of 1000 RETs per mice. Values are indicated as mean  $\pm$  standard deviation, \* $p < 0.05$  as compared to control mice ( $n = 4$ ).

chromatography/tandem mass spectrometry analysis to quantitate the formation of SAFO-DNA adducts *in vivo* after SAFO or safrole exposure may be needed to elucidate the discrepancy between the previous results and our present findings [29].

We observed apparent cytoplasmic vacuolation of hepatocytes around the central vein in the liver from mice receiving 12 doses of SAFO at highest dose tested (60 mg/kg) (Fig. 5). In mice that received 45 mg/kg SAFO, the cytoplasm of some hepatocytes adjacent to the central vein area was finely granular and lighter than that of controls. In mice that received the highest dose (60 mg/kg), there was evidence of cytoplasmic vacuolation or ballooning degeneration immediately surrounding the central vein, whereas, the hepatic cells around the portal vein area remained intact and necrotic cells were not seen. The present study reported that SAFO-treated mice developed liver injury as manifested by a rim of ballooning degeneration of hepatocytes immediately adjacent to the central vein. This characteristic histopathological change induced by SAFO is very different to that seen in safrole-treated mice [43]. Hagan et al. showed that safrole administered to male and female Osborne-Mendel rats at doses of 250, 500, and 750 mg/kg/day for up to 105 days via oral intubation and that safrole administered to Swiss mice at doses of 250 and 500 mg/kg/day for 60 days induced liver changes [43]. Microscopic examination revealed evidence of hepatic cell enlargement, which was usually focal and resulted in the formation of nodules; adenomatoid hyperplasia; cystic necrosis; fatty metamorphosis; and bile duct proliferation [43].

Although the mutagenicity and carcinogenicity of safrole and SAFO have been known for more than 30 years, SAFO has recently been proposed to be a potential candidate for cancer therapy because of its anti-angiogenesis and apoptosis-inducing activity *in vitro*. SAFO has been shown to have anti-angiogenic activity by triggering apoptosis via a mechanism involving the overexpression of Fas, integrin beta4 and P53, attenuation of  $Ca^{2+}$ -independent phosphatidylcholine-specific phospholipase C activity, and the inhibition of intracellular reactive oxygen species generation in vascular endothelial cells [44]. In A549 human cancer cells, SAFO induced apoptosis by up-regulating Fas and FasL [45] and activating caspase-3, -8, and -9 [46]. Furthermore, Yu et al. reported that safrole induced apoptosis in human oral squamous cell carcinoma HSC-3 cells and reduced the size and volume of HSC-3 solid tumors

in a xenograft athymic nu/nu mouse model [47]. These data suggest that SAFO exhibits anti-tumor effects *in vitro* and in mice. Since our data provide the clear evidence for the genotoxicity of SAFO in human cells *in vitro* and in mice, the potential clinical application of SAFO in cancer treatment needs to be critically re-evaluated.

In conclusion, we show clear evidence that SAFO induces significant genotoxic activity *in vitro* and *in vivo*. We first showed that SAFO caused significant dose-dependent increases in cytotoxicity, mean Comet tail moment, and micronucleated binucleated cells in human HepG2 cells. Moreover, we clearly demonstrated that SAFO exhibited significant genotoxic effects in mice, as evidenced by significant dose-dependent increases in mean Comet tail moment in peripheral blood leukocytes and in the frequency of micronucleated reticulocytes in mouse peripheral blood. SAFO also induced liver damage, typically manifested as a rim of ballooning degeneration of hepatocytes immediately adjacent to the central vein. SAFO should not be used directly under normal circumstances; therefore, our findings strongly suggest the need to carefully evaluate the potential use of SAFO in cancer therapy. Our data will provide another potential mechanism for the mutagenicity and carcinogenicity of safrole. Further mechanistic studies on the genotoxicity and mutagenicity of SAFO may be needed to adequately assess its role in safrole carcinogenicity and to help assess the potential health risk to humans due to daily consumption of safrole from edible herbs, spices, and Chinese medicinal plants.

#### Conflict of interest

The authors declare that there are no conflicts of interest.

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