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Genotoxicity of 1-Methylpyrene and 1-Hydroxymethylpyrene in Chinese Hamster V79-Derived Cells Expressing Both Human CYP2E1 and SULT1A1

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1-Methylpyrene (1-MP) is a widespread pollutant that is carcinogenic in animals following metabolic activation. Previous studies have shown that benzylic hydroxylation of 1-MP, catalyzed by multiple CYP isoforms, gives rise to 1-hydroxymethylpyrene (1-HMP), which becomes bioreactive following further metabolism by various sulfotransferase (SULT) isoforms. However, the mutagenic and chromosome damaging effects of 1-MP and 1-HMP in mammalian cells have not been investigated. In this study a Chinese hamster V79-derived cell line expressing both human CYP2E1 and human SULT1A1 was used to investigate the ability of 1-MP and 1-HMP to induce cytotoxicity (using the CCK-8 assay), micronuclei and *Hprt* gene mutations. The role of each enzyme was investigated through co-exposure in the presence of an enzyme inhibitor. We found that at concentrations of 0.5–4 μM and 5–20 μM, under conditions where no reduction in cell viability/growth occurred, 1-HMP and 1-MP induced micronuclei in V79-hCYP2E1-hSULT1A1 cells in a concentration-dependent manner; however, both compounds were inactive in V79 cells. Similarly, they both caused an increase in *Hprt* mutant frequency in V79-hCYP2E1-hSULT1A1 cells in these concentration ranges, with 1-MP impairing cell viability/growth at 10 μM and above in the mutagenicity assay. The compounds were again both inactive in V79 cells. The effects of 1-HMP in V79-hCYP2E1-hSULT1A1 cells were blocked or reduced by addition of pentachlorophenol (PCP), a SULT1 inhibitor; the genotoxicity of 1-MP was significantly reduced by either 1-aminobenotrazole, a CYP2E1 inhibitor, or PCP. The results suggest that human CYP2E1 and SULT1A1 cooperate to activate 1-MP and cause genotoxicity in mammalian cells. Environ. Mol. Mutagen. 00:000–000, 2014.

Key words: CYP2E1; genetically engineered cells; 1-methylpyrene; genotoxicity; sulfotransferase (SULT) 1A1

INTRODUCTION

1-Methylpyrene (1-MP) is common in water and soil, and has been detected in the air of highly populated and urban areas with a high traffic density [Lian et al., 2009]. It is produced from various human sources including cigarette smoke, automobile exhausts, industrial emissions, and pyrolysis of hydrocarbons. It has also been used as a fluorescent probe in laboratories [Mouffouk et al., 2011; Agudelo-Morales et al., 2012]. 1-MP is carcinogenic to rodents, exerting its greatest effects in the liver [Rice et al., 1987]. It has been estimated that the potency of 1-MP carcinogenicity is about one tenth that of benzo(a)-pyrene, a confirmed strong human carcinogen [Hartwig, 2013]; thus, its potency is similar to benzo(a)anthracene. Therefore, the potential carcinogenic hazard of 1-MP to human and ecological health is of significant concern.

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Somatic mutation is a critical mediator of chemical carcinogenesis. As such, assessment of genotoxicity is essential in determining the mechanism of action of carcinogenic compounds. 1-MP has been observed to induce unscheduled DNA synthesis in rat hepatocytes [Rice et al., 1987]. Incubation of 1-MP with rat and human hepatic microsomal preparations, as well as various human CYP isoenzymes (including 1A1, 1A2, 1B1, 2A6, 2E1, and 3A4) individually expressed in Chinese hamster V79 cells, may lead to the production of 1-hydroxymethylpyrene (1-HMP) [Engst et al., 1999], indicating the relevant enzymes involved in metabolic activation of 1-MP. In contrast, microsomal preparations from CYP-deficient V79 cells are unable to activate 1-MP [Engst et al., 1999].

In addition to the important roles of various CYPs in metabolism of 1-MP, rat sulfotransferases (SULT) 1A1, 1C1, and 2A3 are capable of activating 1-HMP to a genotoxic metabolite (as indicated by abruptly increased DNA repair synthesis) [Andrae et al., 1999]. A recent work confirms that additional isoforms of rat SULTs, including orphan forms such as rat SULT2A3 and SULT5A1, are capable of activating 1-HMP to produce mutagenic responses in *Salmonella typhimurium*. Cytosolic preparations from tissues of transgenic mice that express human SULT1A1/2 significantly enhance the mutagenicity of 1-HMP in bacteria compared to wild-type mice [Bendadani et al., 2014a]. Furthermore, 1-MP appears to form DNA adducts in the liver, lungs, and kidneys of both mice and rats *in vivo*, with increased adduct formation in transgenic mice expressing human SULT1A1 and 1A2 [Bendadani et al., 2014b]. In the latter studies, 1-sulfooxymethylpyrene (1-SMP), the proposed ultimate carcinogen of 1-MP, was found in the serum of all treated animals [Bendadani et al., 2014b].

An existing gap is empirical data to understand the mutagenicity or chromosome damaging activities of 1-MP, or its intermediate metabolite 1-HMP, in mammalian cells. In particular, knowledge of the genotoxic potential of 1-MP under combined activation by both phase I and II enzymes is required. In this study, we used a V79-derived cell line that was genetically engineered to express both human CYP2E1 and human SULT1A1 (V79-hCYP2E1-hSULT1A1), to evaluate the ability of 1-MP and 1-HMP to induce cytotoxicity, micronuclei, and gene mutations. The relevance of these enzymes in the biotransformation of both the parent compound and its metabolite, and the resulting effects on cytotoxicity and genotoxicity, were assessed through the use of specific inhibitors for each enzyme, and through comparison with control V79 cells that do not express the enzymes.

MATERIALS AND METHODS

Chemicals

1-Aminobenzotriazle (ABT), dimethylsulfoxide (DMSO), 1-MP, *N*-nitrosodimethylamine (NDMA), 6-thioguanine, and 2-nitropropane (2-NP) were purchased from Sigma Aldrich (St. Louis, MO). 1-HMP was purchased from Tokyo Chemical Industry (Tokyo, Japan). Cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan), and pentachlorophenol (PCP) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Ethyl methanesulfonate (EMS, purity of 98.5%) was purchased from J & K Chemical (Suzhou, China). NDMA and 2-NP were dissolved and dispersed in pure water, respectively, EMS and CCK-8 were applied directly to the cell culture, while 1-MP, 1-HMP, ABT, and PCP were all dissolved in DMSO before exposure.

Cell Lines

The V79 cell line was purchased from Shanghai Fuxiang Biotech. (Shanghai, China), and the V79-hCYP2E1-hSULT1A1 cell line was a generous gift from Dr. Hansruedi Glatt (Nuthetal, Germany). The latter cell line was constructed from the V79-hCYP2E1 cell line (a V79-Mz-derived cell line that expresses the wild type human CYP2E1 following genetic engineering) [Schmalix et al., 1995], by introducing wild-type SULT1A1. The established cell line expresses both enzymes stably; however, the level of human CYP2E1 is 3–4 times greater than the parental V79-hCYP2E1 cells. To minimize the spontaneous mutant frequency prior to mutagenicity assays with V79-hCYP2E1-hSULT1A1 cells, we removed pre-existing mutants by culturing in HAT medium (medium supplemented with 0.4 μM aminopterin, 0.1 mM hypoxanthine, 0.016 mM thymidine, and 0.1 mM glycine) for three passages [Liu and Glatt, 2010]. ABT (20 μM) and/or PCP (10 μM), inhibitors of CYP2E1 and SULT1A1, respectively, were used to observe the role of each enzyme in the effects of 1-MP and 1-HMP in V79-hCYP2E1-hSULT1A1 cells [Liu and Glatt, 2008]. Cells were cultured in Dulbecco's modification of Eagle's medium (Gibco) supplemented with 7% fetal bovine serum (Gibco), 100 IU/mL penicillin G, and 100 μg/mL streptomycin, at 37°C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity (CCK-8) Assay

The cytotoxicity of 1-MP and 1-HMP was determined in both cell lines using disulfonated tetrazolium salt (CCK-8) as a chromogenic indicator for NADH and cell viability, based on a well established method [Ishiyama et al., 1997]. Both of the exposure-recovery regimens that were applied in the micronucleus or *Hprt* mutation assays were used to evaluate cytotoxicity.

For CCK-8 assays adapted for the micronucleus assay, cells were inoculated on 96-well plates at a density of 6.8×10^3 cells/well in 100 μL of culture medium. 1-MP or 1-HMP was added at varying concentrations (or DMSO as the vehicle) to each well 24–36 hr post-inoculation. Six wells were used for each treatment. At 48 hr, 10 μL of CCK-8 was added to each culture and cells were incubated for 2 hr. Optical density at 450 nm (OD_{450}) was then measured on a microplate reader (BioRad Model 680). To evaluate the role of CYP2E1 or SULT1A1 in the induced responses in V79-hCYP2E1-hSULT1A1 cells, cells were exposed to either ABT (20 μM) or PCP (10 μM) from 22 to 48 hr (medium changed at 24 and 36 hr for addition and removal of 1-MP or 1-HMP, respectively). DMSO was limited to a final concentration of 0.04% (v:v).

For CCK-8 assays adapted to the mutagenicity assay, cells were inoculated on 96-well plates at a density of 2×10^3 cells/well, and exposed to each compound 24–48 hr post-inoculation (six cultures per treatment group). After removal of the test compound, each culture was further incubated for 2 d and then subject to CCK-8 staining and measurement of OD_{450}. To investigate the role of each enzyme, ABT and PCP at the indicated concentrations were added to each culture from 22 hr to 4 d (medium changed at 24 and 48 hr for addition and removal of 1-MP or 1-HMP, respectively).

Micronucleus Test

The micronucleus test was carried out as previously described [Liu et al., 1998; Deng et al., 2011]. Either V79 or V79-hCYP2E1-hSULT1A1 cells

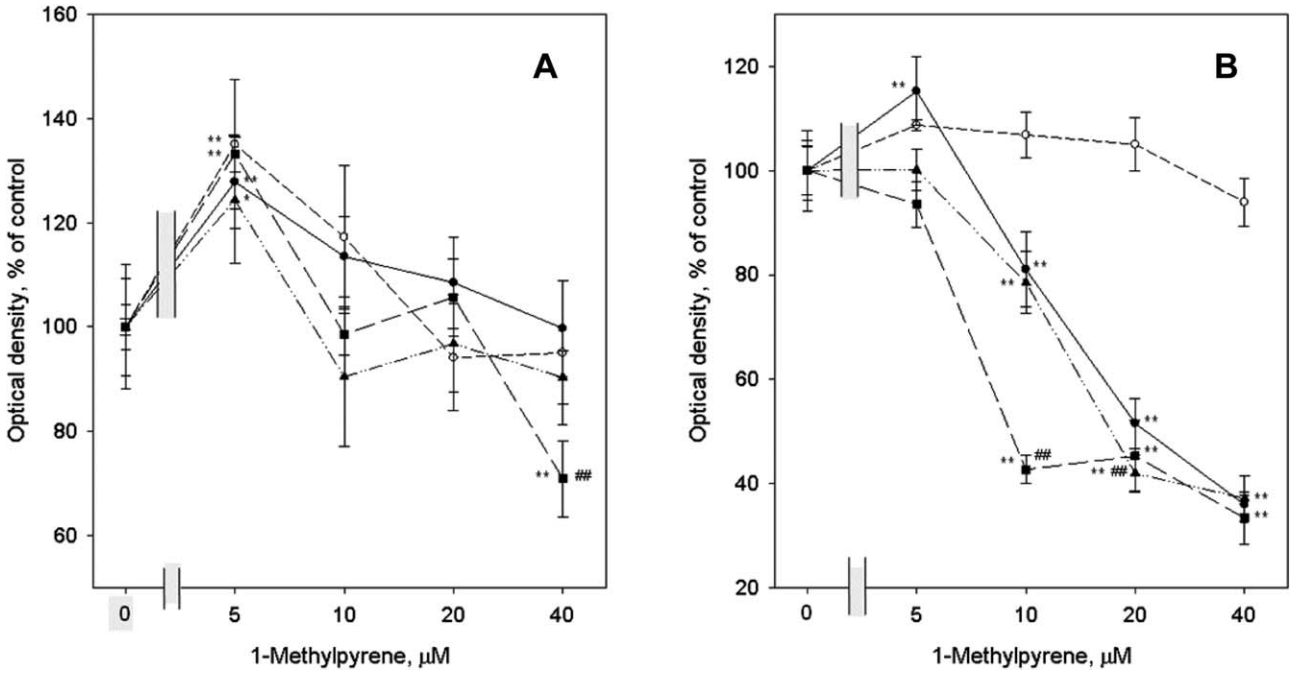

Fig. 1. Cytotoxicity of 1-MP (CCK-8 assay) under the exposure/recovery schedule of 12 hr/12 hr (A) and 1 d/2 d (B) in V79 control cells (○) or V79-hCYP2E1-hSULT1A1 cells, alone (●) and in combination with ABT (20 μM, ▲) or PCP (10 μM, ■). Data are means and S.D. of six replicates; $^{**}P < 0.01$, by ANOVA, compared with the negative control; $^{\#\#}P < 0.01$, by ANOVA, comparison was made between a treatment with and without a modulator.

were inoculated at a density of 3×10^5 /flask (12.5-cm^2) and exposed to 1-MP or 1-HMP at varying concentrations from 24 to 36 hr. Three replicates were used per treatment group. At 48 hr cells were harvested, made hypotonic by treatment with 0.075 M of KCl in a water bath at 37°C for 5 min, fixed in methanol:acetic acid (3:1) for 10 min, resuspended in methanol:acetic and dropped onto glass slides, and finally stained with Giemsa stain. For inhibition of CYP2E1 or SULT1A1 in V79-hCYP2E1-hSULT1A1 cells, ABT (20 μM) or PCP (10 μM) was added to each culture from 22 to 48 hr. The final concentration of DMSO was limited to 0.04% (v:v). The CYP2E1- and SULT-dependent promutagens NDMA (100 μM) and 2-NP (5 mM) served as positive controls in V79-hCYP2E1-hSULT1A1 cells. EMS (5 mM) was used as a positive control for tests in V79 cells. Under microscopy at 1,000 × magnification (oil lens), 1,000 cells (with an intact cell membrane and appropriately differential staining of nucleus and cytoplasm) from each culture were scored to determine the frequency of micronucleated cells (cells with one or more micronuclei present in the cytoplasm without overlapping the main nucleus) by an experienced scorer.

Hprt Mutation Assay

Forward mutations at the *Hprt* locus, which are measured as cells acquiring resistance to 6-thioguanine, were used to examine effects on gene mutations. The assay was carried out as described previously [Liu et al., 2008] with minor modifications. Briefly, 1.0×10^6 of V79 or V79-hCYP2E1-hSULT1A1 cells were inoculated in 25 mL medium onto 150-cm^2 Petri dishes. At 24 hr, cells were exposed to 1-MP and 1-HMP at varying concentrations. The direct acting mutagen EMS (1.6 mM) was used as the positive control in V79 cells. In V79-hCYP2E1-hSULT1A1 cells, NDMA (100 μM) and 2-NP (5 mM) served as positive controls. Duplicate cultures were set up for each treatment. Test compound exposure was carried out for 24 hr (1 d exposure), and then the medium was replaced with fresh medium. At 4 d (subsequent to 2 d recovery), the cells were detached by treatment with trypsin, harvested, and counted using a hemocytometer. Cell counts may be used as a measure of the cytotoxicity of a test compound, by comparing cell numbers in treatment groups to those in the vehicle control. The cells were subcultured for 3 days, and then subcultured again using medium supplemented with 6-thioguanine (7 μg/mL) for selection of mutants (10^6 cells per 150-cm^2 Petri dish, four dishes). Subsets of cells were subcultured in normal medium in parallel to determine the colony-forming efficiency (100 cells per 12.5-cm^2 Petri dish, three dishes). After 8 d, the cultures were fixed in ethanol and stained with Giemsa stain; the cell colonies were counted and mutant frequencies calculated from the averaged values for each initial culture. To investigate the role of each expressed human enzyme in the mutagenicity of 1-MP and 1-HMP in V79-hCYP2E1-hSULT1A1 cells, co-exposures with ABT (20 μM) or PCP (10 μM) were conducted for some cultures from 22 hr to 4 d.

Statistical Analysis

The relative cell growth and viability detected by the CCK-8 method for each treatment (expressed as percent of OD_{450} values in each treatment over those in the vehicle control) was compared with the vehicle control using an ANOVA. The frequency of micronucleated cells in each treatment was expressed as a mean ± S.D. The frequencies of micronucleated cells in treatment groups were compared with the vehicle control group, or comparisons were made between treatments with and without an enzyme inhibitor, using Student's t-tests. The mutant frequencies in each treatment were expressed as means and range of variation; duplicates were combined for a total frequency of mutants from each treatment (i.e., becoming quantal bioassay data), and compared with the vehicle control using a χ^2 analysis.

RESULTS

Cytotoxicity of 1-MP and 1-HMP in V79 and V79-hCYP2E1-hSULT1A1 Cells

Using an exposure-recovery schedule of 12 hr + 12 hr (which corresponds to the design used in the micronucleus

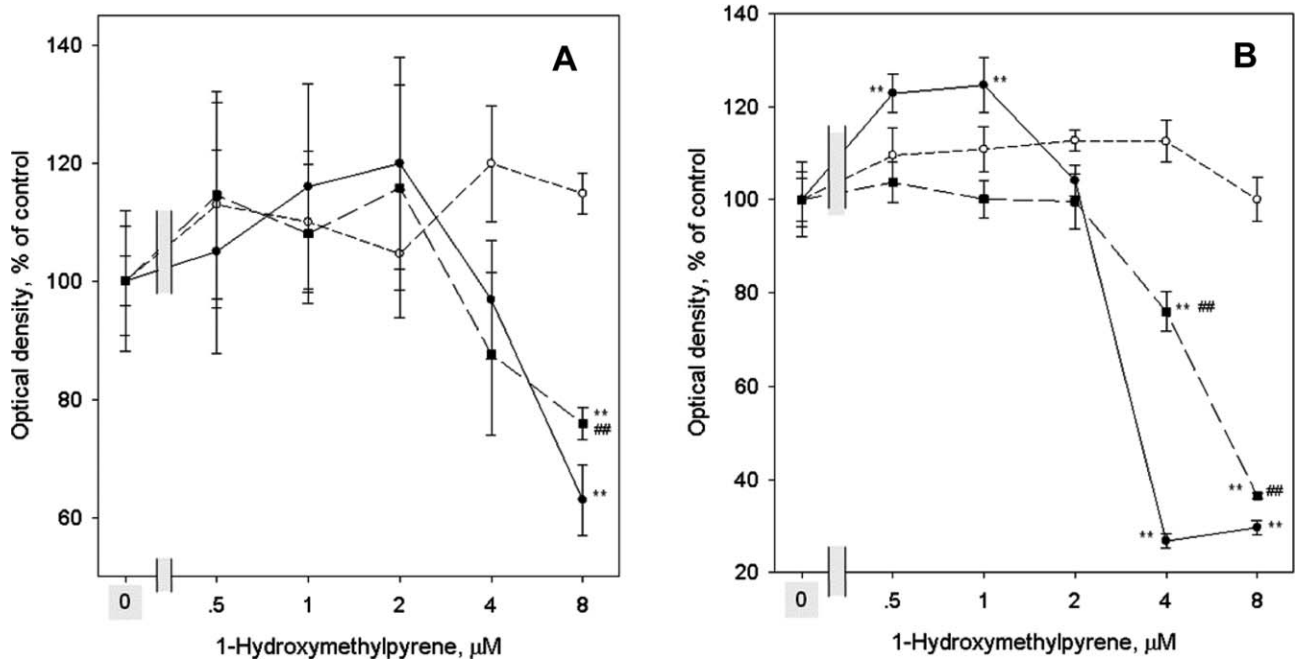

Fig. 2. Cytotoxicity of 1-HMP (CCK-8 assay) under the exposure/recovery schedule of 12 hr/12 hr (A) and 1 d/2 d (B) in V79 control cells (○) or V79-hCYP2E1-hSULT1A1 cells, alone (●) and in combination with PCP (10 μM, ■). See legend of Figure 1.

test), 1-MP did not reduce cell viability/growth at concentrations ranging from 5 to 40 μM (the limit of solubility) in either V79 or V79-hCYP2E1-hSULT1A1 cells (Fig. 1A). Rather, exposure to 1-MP caused an increase in relative OD_{450} at 5 μM in both of these cell lines, with no apparent effect of addition of ABT and PCP (inhibitors of CYP2E1 and SULT1A1, respectively) in the presence of 1-MP in the V79-hCYP2E1-hSULT1A1 cells. These results suggest that there was a hormetic effect of 1-MP in the cells that was independent of enzyme expression. Co-exposure of V79-hCYP2E1-hSULT1A1 cells to PCP led to a moderate reduction in OD_{450} when 1-MP reached 40 μM, indicating a combined effect of PCP (10 μM) and 1-MP (40 μM) on cytotoxicity.

1-MP had no effect on cell growth/viability using a 1 d + 2 d exposure-recovery schedule in V79 cells (Fig. 1B); however, notable differences in cytotoxicity were found in the V79-hCYP2E1-hSULT1A1 cell line. 1-MP induced an increase in OD_{450} at 5 μM in V79-hCYP2E1-hSULT1A1 cells. The magnitude of this effect was smaller than that observed in the 12 hr + 12 hr schedule. This hormetic effect did not occur in either the V79 cells or the V79-hCYP2E1-hSULT1A1 cells cotreated with ABT or PCP. However, 1-MP caused a decrease in OD_{450} values in V79-hCYP2E1-hSULT1A1 in a concentration-dependent manner beginning at the 10 μM concentration. Co-exposure with ABT slightly potentiated the cytotoxicity of 1-MP at 20 μM. Similarly, PCP potentiated the cytotoxicity of 1-MP at 10 μM.

1-HMP had no effect on the OD_{450} of V79 cells under a 12 hr + 12 hr exposure-recovery period across the concentrations tested (Fig. 2A). In contrast, a decrease in OD_{450} was noted for the V79-hCYP2E1-hSULT1A1 cells at 8 μM; however, co-exposure with PCP moderately reduced the cytotoxicity of 1-HMP at 8 μM ($P < 0.01$), suggesting the involvement of human SULT1A1 in activating 1-HMP. Similarly, the 1 d + 2 d exposure-recovery regimen showed no effect of 1-HMP in the V79 cells (Fig. 2B); however, 1-HMP reduced the viability/growth of the V79-hCYP2E1-hSULT1A1 cells at 4 and 8 μM. In addition, exposure to lower concentrations (0.5 and 1 μM) of 1-HMP increased cell growth in V79-hCYP2E1-hSULT1A1 cells. This effect was not observed in V79 cells or in V79-hCYP2E1-hSULT1A1 cells co-exposed to PCP, indicating the potential role of human SULT1A1 in the hormesis observed following 1-HMP exposure. Co-exposure with PCP greatly reduced the cytotoxicity of 1-HMP at 4 μM. This effect persisted at 8 μM but was not as large. These data provide further support for the involvement of human SULT1A1 in activating 1-HMP.

To validate the CCK-8 assay as a measure of cell viability/growth for the cells used in the present study, cell counts for V79-hCYP2E1-hSULT1A1 cells treated with 1-MP at various concentrations were determined during the first subculturing in the mutagenicity assay. The correlation between cell counts and the CCK-8 assay was evaluated. Cell counts following exposure of V79-hCYP2E1-hSULT1A1 to 1-MP (Fig. 3A) were highly correlated with the OD measures in the CCK-8 assay (Fig. 1B). Linear regression of cell counts with CCK-8 assay results yielded

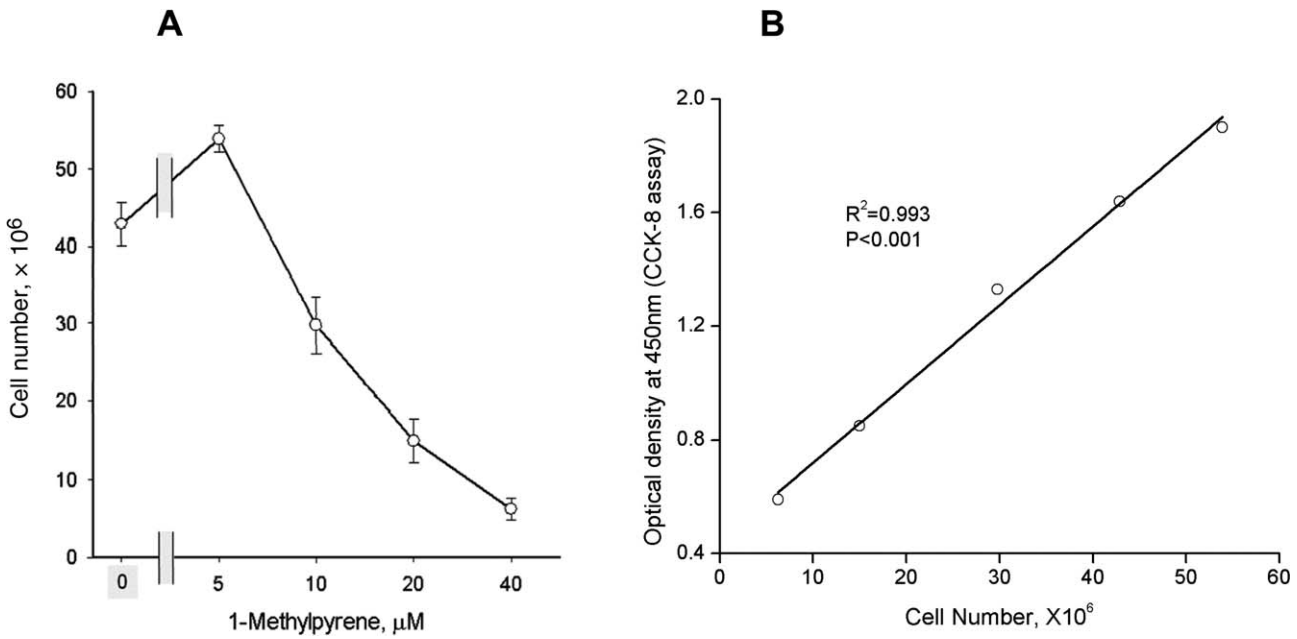

Fig. 3. Cytotoxicity of 1-MP in V79-hCYP2E1-hSULT1A1 cells indicated by cell numbers at the first subculturing (4 d) of the mutagenicity assay (A) and its linear correlation with that by CCK-8 assay (B). Cells were exposed to 1-MP alone and data are means and range of variation of duplicate cultures (A). Data of CCK-8 assay were taken from Figure 1B.

a correlation coefficient of 0.993 ($P < 0.001$). The results suggest that the CCK-8 assay effectively represents cell number (division) changes induced by chemicals.

Induction of Micronuclei by 1-MP and 1-HMP in V79 and V79-hCYP2E1-hSULT1A1 Cells

Cell counts for V79 and V79-hCYP2E1-hSULT1A1 cells upon harvest at 48 hr for the micronucleus test (counted using a hemocytometer) were 2.80 ± 0.13 and 2.44 ± 0.26 ($\times 10^6$), respectively ($P > 0.05$ by Student's t-test). These numbers indicate an approximate 8–9 times increase in cell number from inoculation, equivalent to about three cell doubling cycles (both cell lines have a doubling time of about 12 hr). 1-MP (5–20 μM) had no effect on the frequency of micronucleated cells in V79 cells (Fig. 4A); however, a concentration-dependent increase in the frequency of micronucleated cells was observed in V79-hCYP2E1-hSULT1A1 cells. Similarly, while 1-HMP was inactive in V79 cells, it caused a significant increase in the frequency of micronucleated cells in V79-hCYP2E1-hSULT1A1 cells (Fig. 4B).

To test the validity of the micronucleus test, we used the direct mutagen EMS (5 mM) as a positive control for V79 cells. EMS significantly increased the frequencies of micronucleated cells; moreover, it induced gene mutations in these cells at a concentration of 1.6 mM (legends of Figs. 4 and 5). In V79-hCYP2E1-hSULT1A1 cells, NDMA (100 μM) and 2-NP (5 mM) slightly increased the frequency of micronucleated cells (legend of Fig. 4), which was similar to our previous observation [Deng et al., 2011]; an apparently stronger induction of gene mutations at the *Hprt* locus than of micronucleus formation was observed with NDMA and 2-NP (legend of Fig. 5), indicating the relative predominance of gene mutations induced by these promutagens.

Effects of Enzyme Inhibitors on Induction of Micronuclei by 1-MP and 1-HMP in V79-hCYP2E1-hSULT1A1 Cells

As shown in Figure 4A, co-exposure of V79-hCYP2E1-hSULT1A1 cells to ABT (20 μM) or PCP (10 μM) significantly reduced the induction of micronuclei by 1-MP at every concentration. Indeed, PCP blocked the induction of micronuclei by 1-MP at 5 μM completely, and significantly decreased 1-MP-induced micronuclei at higher concentrations. Co-exposure with ABT also appeared to cause a decrease in micronucleus induction by 1-MP at each concentration. However, the largest effect was observed in V79-hCYP2E1-hSULT1A1 cells following 1-HMP exposure (Fig. 4B), where a tremendous reduction in induced frequency of micronucleated cells occurred as a result of co-exposure with PCP (i.e., completely blocked with 1-HMP at 0.5 and 1 μM, and greatly reduced at 2 and 4 μM of 1-HMP). The above results provide an evidence to support that human CYP2E1 and human SULT1A1 are capable of cooperatively activating 1-MP to a mutagenic metabolite, whereas 1-HMP may only require SULT1A1 to be activated.

Induction of *Hprt* Mutations by 1-MP and 1-HMP in V79 and V79-hCYP2E1-hSULT1A1 Cells

Cell numbers of V79 and V79-hCYP2E1-hSULT1A1 cells increased from 1×10^6 (on inoculation) to

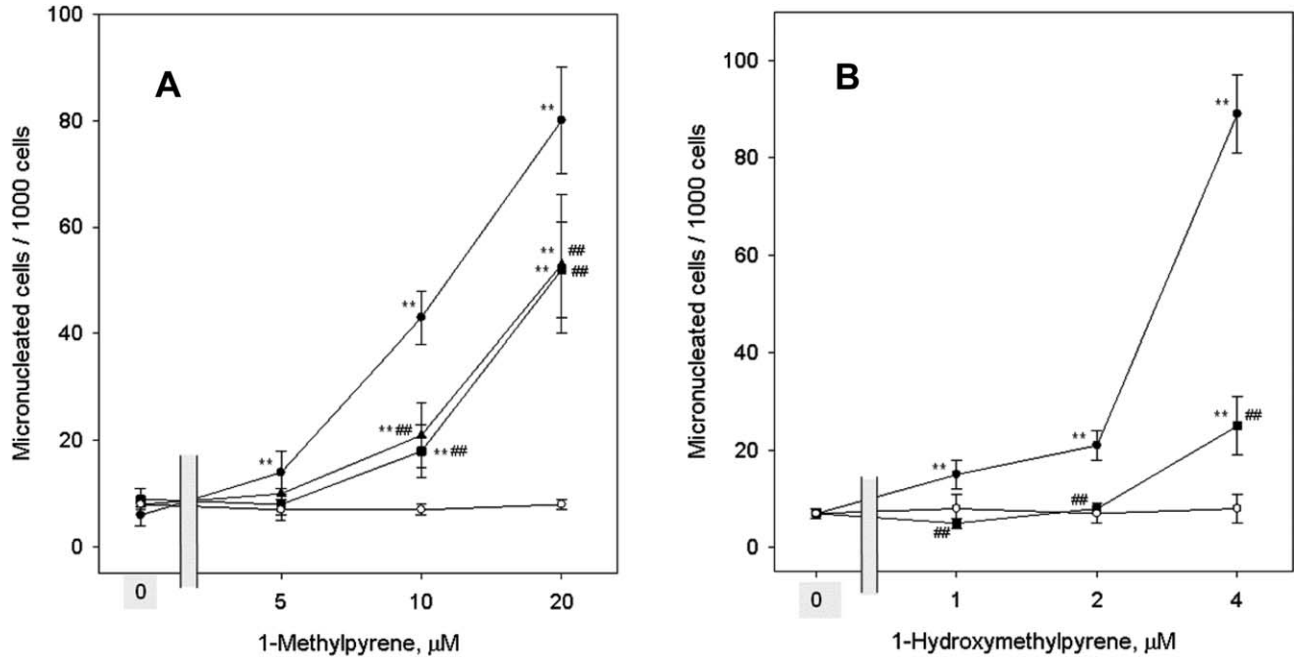

Fig. 4. Induction of micronuclei by 1-MP (A) and 1-HMP (B) in V79 (○) and V79-hCYP2E1-hSULT1A1 cells, alone (●) and in combination with ABT (20 μM, ▲) or PCP (10 μM, ■). Data are means ± S.D. of three replicates. Statistical analysis was performed by Student's t-test, $^{**}P < 0.01$, compared with the negative control; $^{\#\#}P < 0.01$, comparison was made between a treatment with and without a modulator. As positive controls, EMS (5 mM) elevated the frequency of micronucleated cells in V79 cells to 49 ± 7 (‰), while NDMA (100 μM) and 2-NP (5 mM) elevated the frequency in V79-hCYP2E1-hSULT1A1 cells to 19 ± 3.6 and 15 ± 1.3 (‰), respectively.

45.8 ± 4.2 and 42.9 ± 2.8 (× 10^6) (on 4 d), respectively, indicating over five cell doubling cycles up to the first subculturing in the *Hprt* mutagenicity assay. 1-MP did not induce gene mutations in V79 cells (Fig. 5A). In contrast, 1-MP caused a concentration-dependent increase in gene mutations in V79-hCYP2E1-hSULT1A1 cells. Mutant frequency began to steeply increase from 5 μM. Co-exposure with ABT (20 μM) significantly reduced the frequency of mutants induced by 1-MP; indeed, ABT almost entirely blocked the mutagenicity of 1-MP at lower (5 and 10 μM) concentrations, and significantly reduced the effect of 1-MP at 20 μM. PCP (10 μM) also inhibited the induction of gene mutations by 1-MP at various concentrations. Exposure to 1-HMP had no effect on mutant frequency in V79 cells either (Fig. 5B). However, 1-HMP caused a concentration-dependent increase in mutant frequency in V79-hCYP2E1-hSULT1A1 cells. This increase was reduced by PCP co-exposure with 1-HMP at higher concentrations (2 and 4 μM).

DISCUSSION

The present study provides evidences for cooperative activation of 1-MP by human CYP2E1 and SULT1A1. This finding was consistent for both the micronucleus and *Hprt* gene mutation assays. 1-MP induced micronuclei in V79-hCYP2E1-hSULT1A1 cells at concentrations where cell viability/growth was unreduced; however, gene mutations were induced by 1-MP primarily in the presence of some degree of cytotoxicity, which was likely due to the extended exposure period for this assay. 1-HMP induced both micronuclei and gene mutations in V79-hCYP2E1-hSULT1A1 cells at concentrations where no reductions in cell viability/growth were observed. 1-HMP was approximately 10 times more potent than its parental compound (1-MP). Various findings from this study suggest the ability of human CYP2E1 in cooperation with SULT1A1, and SULT1A1 on its own, to activate 1-MP and 1-HMP and thus produce cytotoxic and mutagenic effects, including: (1) the absence of cytotoxic and genotoxic effects of 1-MP and 1-HMP in V79 cells; (2) the induction of micronuclei and gene mutations in response to these two test compounds in V79-hCYP2E1-hSULT1A1 cells; and (3) the significant reduction in these effects by co-exposure with enzyme inhibitors. Furthermore, the hormetic effects of 1-HMP in V79-hCYP2E1-hSULT1A1 cells were completely inhibited by PCP, and absent in V79 cells, which also supports a role for human SULT1A1 in the observed effects of 1-HMP. 1-MP also produced a hormetic effect, but in both cell lines, and particularly under the shorter-term (12 hr + 12 hr) exposure and sampling design. ABT and PCP failed to inhibit this effect in V79-hCYP2E1-hSULT1A1 cells, suggesting that this

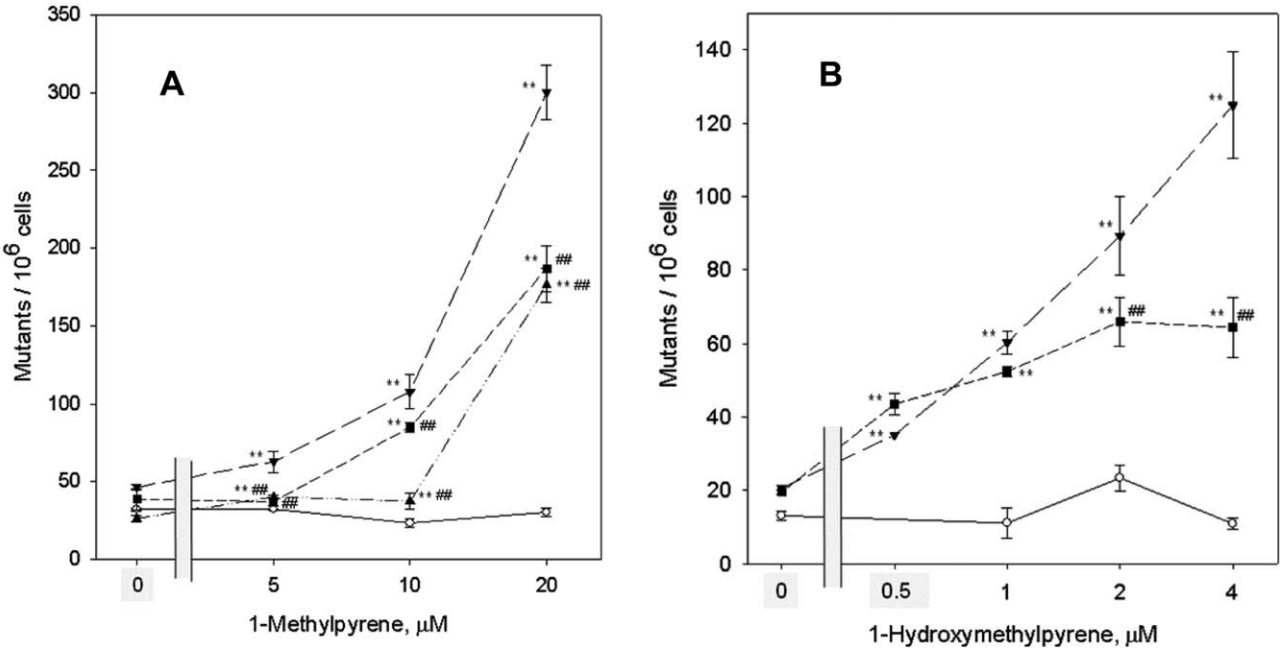

Fig. 5. Induction of gene mutations at the *Hprt* locus by 1-MP (A) and 1-HMP (B) in V79 (○) and V79-hCYP2E1-hSULT1A1 cells, alone (▼) and in combination with ABT (20 μM, ▲) or PCP (10 μM, ■). Values are means and range of variation for two cultures. As a direct mutagen (positive control), EMS (1.6 mM) elevated the gene mutations in V79 cells from 3 ± 1.5 to 345 ± 8 per 10^6 cells; NDMA (100 μM) and 2-NP (5 mM), CYP2E1- and SULT-dependent promutagens, gave rise to 871 ± 150 and 219 ± 12 mutants per 10^6 cells in V79-hCYP2E1-hSULT1A1 cells, respectively. When χ^2 analysis was made, data from the duplicate cultures were joined together as a total frequency; $^{**}P < 0.01$, compared with the negative control; $^{\#\#}P < 0.01$, comparison was made between a treatment with and without a modulator.

hormesis is independent of these enzymes. This difference in the hormetic effects induced by 1-MP and 1-HMP is a novel finding and further mechanistic insight is required to understand the mechanisms underlying these effects.

While the genotoxicity of 1-HMP is about 10 times greater than 1-MP, the latter is actually comparable to NDMA, an established and strong CYP2E1-dependent mutagen (Figs. 4 and 5 including legends). Therefore, our results suggest that 1-MP and 1-HMP are strong mutagens in mammalian cells that depend on combined metabolic activation by both phase I and/or phase II biotransformation enzymes such as CYP2E1 and/or SULT1A1. The above results are concordant with a recent report showing that cytosolic preparations from Sult1a1 knockout mice exhibit a 96% decrease in their ability to activate 1-HMP and produce *Salmonella* reverse mutants [Bendadani et al., 2014a]. The results also demonstrate an important role for Sult1a1 in producing hepatic and renal DNA adducts in 1-HMP-exposed mice. Finally, the study found a 100-fold increase in pulmonary activation of 1-HMP by transgenic human SULT1A1/2 [Bendadani et al., 2014a].

Oxidation of 1-MP at the benzylic position of 1-MP by various CYP isoenzymes is established. Other CYPs, including human CYP1A1, 1B1, and 1A2, appear to be much more active than human CYP2E1 in metabolizing 1-MP [Engst et al., 1999]. Human liver and other tissues express a wide spectrum of these enzymes [Leclerc et al., 2010; Liu et al., 2014] as well as various SULT isoenzymes [Glatt and Meinl, 2004; Teubner et al., 2007]. The choice of only V79-hCYP2E1-hSULT1A1 cells in the present study was based on the availability of cell lines expressing relevant isoenzymes. We may expect a largely different circumstance in human hepatocytes for activation of 1-MP. Many CYP isoforms [Engst et al., 1999] on the endoplasmic reticulum are able to bind to 1-MP, thus catalyzing the benzylic hydroxylation in concert; the 1-HMP that is formed may be activated further by human SULT1A1 and other SULT isoforms [Oda et al., 2012; Bendadani et al., 2014a] in the cytoplasm to form the ultimate mutagen 1-SMP. Therefore, activation of 1-MP by consecutive metabolizing steps to its ultimate toxicant by multiple isoforms of both phase I and II enzymes may be a critical process for its carcinogenesis in animals and even humans. Use of a human primary hepatocyte culture to analyze the roles of natively expressed CYPs and SULTs in activating 1-MP may produce a more quantitative understanding of the pathways for metabolic activation of 1-MP and its hazardous effects in humans. Application of such cells is highly recommended for future studies. Alternatively, S9 fractions prepared from ethanol-induced rats (wherein CYP2E1 was specifically induced) supplemented with both a NADPH-generating system and PAPS (3′-phosphoadenosine-5′-phosphosulfate), may also be effective in activating 1-MP for the

assessment of mutagenicity. However, the following shortcomings may compromise the use of S9 as a model in the study of 1-MP-induced mutagenicity: (1) the biotransformation enzymes involved are from rat species that may show varied substrate specificity compared to homologous human enzymes; (2) the relevant metabolic reactions must happen in the culture medium rather than the intact cells, thus the anion ($SO4^{2-}$)-carrying metabolite (1-SMP) that is formed may be less readily transported through the cell membranes into the tested cells.

In conclusion, although benzylic hydroxylation and subsequent sulfo-conjugation have been established as the metabolic activating pathway for 1-MP, we have demonstrated the mutagenic and chromosome damaging activities of 1-MP and 1-HMP in mammalian cells for the first time, and confirmed the roles of human CYP2E1 and human SULT1A1 in these effects. Our results support the importance of continued studies on the carcinogenicity and mutagenicity of 1-MP in humans as well as regulation of its environmental exposure. Particularly, additional studies on the metabolic activation of 1-MP and the resultant hazardous effects produced in human tissue-based models (e.g., human primary hepatocytes that express other CYPs that are more capable of activating 1-MP than CYP2E1) are required to determine the relevance of this work to the effects of 1-MP on human health.

AUTHOR CONTRIBUTIONS

Dr. Yungang Liu designed the study. Hao Jiang, Yanmei Lai, Yungang Liu, Keqi Hu, Danxun Chen, and Bixuan Liu performed the experiments. They worked together in analyzing the obtained data, and in the presentation of the results. Dr. Yungang Liu wrote the manuscript. All authors approved the final manuscript.

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