Role of exposure/recovery schedule in micronuclei induction by several
promutagens in V79-derived cells expressing human CYP2E1 and SULT1A1

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ABSTRACT

The standard procedure for the micronucleus test in cell lines requires a short exposure (≤ 0.5 cell cycle) to the test compounds followed by a long recovery (≥ 1.5 cell cycle), and in case of negative or equivocal results, a second test with extended exposure (≥ 2 cell cycles) without or with a recovery time. In general the two procedures are advantageous for detecting clastogens and aneugens, respectively. However, whether the recommended procedures apply to micronucleus tests with promutagens in cell lines genetically engineered for expressing biotransformation enzymes has not been identified. In this study, several promutagens dependent on cytochrome P450 (CYP) 2E1 and/or sulfotransferase (SULT) 1A1 were used in the micronucleus test in a Chinese hamster V79-derived cell line expressing human CYP2E1 and SULT1A1 (V79-hCYP2E1-hSULT1A1), with varying exposure/recovery schedules: 3 h/21 h, 6 h/18 h, 12 h/12 h, 18 h/6 h, and 24 h/0 h, in comparison with known clastogens and aneugens in V79 control cells. The results showed peaked micronuclei induction by mitomycin C and bleomycin (clastogens) at the 12 h/12 h schedule, while colchicine and vinblastine (aneugens) showed strongest effect at 24 h/0 h. Catechol and trihydroxybenzene (activated by CYP2E1) induced micronuclei most strongly at 6 h/18 h, whereas somewhat longer exposures were optimal for hydroquinone, another compound activated by CYP2E1. 1-Hydroxymethylpyrene (activated by SULT1A1) and 1-methylpyrene (activated sequentially by CYP2E1 and SULT1A1) produced the highest response with the 18 h/6 h treatment regimen. Moreover, mitotic arrest by 1-hydroxymethylpyrene was observed in V79-hCYP2E1-hSULT1A1 cells but not in V79 cells, and 1-methylpyrene arrested mitosis in V79-hCYP2E1-hSULT1A1 more strongly than in V79 cells. Our study suggests that intracellular bioactivation of promutagens may not delay the induction of micronuclei in the present model, and 1-methylpyrene and 1-hydroxymethylpyrene may be activated to mitosis-arresting metabolites.

Key words: Aneugen; clastogen; CYP2E1; sulfotransferase (SULT); micronucleus; exposure/recovery schedule

1. Introduction

The micronucleus test is a cytogenetic method with clear endpoints of detection: both chromosome loss and breakage. It was first established as an *in vivo* model to test the genotoxic potential of xenobiotics by observation of bone marrow erythrocytes [1]. The *in vivo* micronucleus test soon became more commonly applied than the classical *in vivo* chromosome aberration test, due to the accordance of results from the two tests to each other and the apparent convenience of the micronucleus test over the chromosome aberration test. Later, *in vitro* micronucleus tests were established in both human lymphocytes and immortalized cell lines [2,3]. Again, the *in vitro* micronucleus test appears to be consistent with the *in vitro* chromosome aberration test in evaluating the genotoxic potentials of various chemicals [4,5], and less labor and time are required for carrying out the micronucleus test. Therefore, the micronucleus tests (both *in vitro* and *in vivo*) have been included in the standard battery of genetic toxicity tests, formulated by various organizations such as Organization for Economic Co-operation and Development (OECD) [6], International Conference on Harmonization (ICH) [7], and US Environmental Protection Agency (EPA) [8], for more than 30 years. In fact, the *in vitro* micronucleus test is commonly used as one of the first-line screening genotoxicity tests with new chemicals. In an effort to validate the *in vitro* micronucleus test, international working groups have proposed essential treatment schedules for tests with cell lines: the first experiment should be done with a short exposure (3–6 h) to the test chemical and a long recovery period (2 cell cycle length or longer after the beginning of exposure) before sampling, and in case of negative results an additional experiment with a long exposure (e.g., 2 cell cycles) without or with a recovery period has to be followed [9,10]. It is clear that the combination of the two treatment schedules in the *in vitro* micronucleus test helps increasing the sensitivity of genotoxicant detection and minimizing false negative results. Furthermore, experiments in mammalian and human cell lines suggest that a long exposure and/or short recovery period is favorable for detection of aneugen-induced micronuclei, while a short exposure and extended recovery is optimal for detection of clastogens [6,11,12].

Since numerous genotoxic chemicals require metabolic activation before exerting their effects, use of metabolic activating systems in the *in vitro* genotoxicity assays is a common strategy for prevention from false negative results due to insufficient activities of metabolic enzymes expressed in standard test cells [13]. Hepatic postmitochondrial fraction from rats treated with the enzyme inducer Aroclor 1254 supplemented with an NADPH-generating system (S9 mix) is now nearly universally used in most standard *in vitro* genotoxicity tests [13]. Its major advantage is in its technical simplicity and easy standardization. However, one has to be aware it only comprises activities of a limited number of enzymes. Primarily, it is a rich source for certain

cytochromes P450 (CYP), in particular 1A, 2B, 2C, 2D and 3A forms [13,14]. Yet, it is now evident that CYP forms that are low in standard S9 mix, such as CYP1B1 and 2E1, as well as various non-CYP enzymes are involved in the bioactivation of a substantial number of carcinogens [15]. Next to CYPs, sulfotransferases (SULTs) were most often found to be critically involved in bioactivation reactions [15]. This activity is not taken into account in standard S9 mix, due to insufficient levels of the cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS); and even if S9 fraction is supplemented with PAPS, the formed sulfo-conjugate product may not readily enter the target cells owing to the negative charges carried by the product [16,17]. This may lead to the unavailability of the reactive metabolites to the intracellular target molecules and thus may produce false negative results [13,17]. The second shortcoming of S9 mix is its limited stability during the incubation. Various enzymes are losing activity and lipid peroxidation may lead to cytotoxic, or even genotoxic, products. For these reasons, only short exposure periods are possible when using S9 mix. Consequently, the use of extended exposure periods in the *in vitro* micronucleus assay is limited to the direct test according to the established guidelines [6–8].

Human primary hepatocyte cultures may express various biotransformation enzymes, however, they are not feasible for the detection of transmittable gene mutations or chromosome damage, since they do not divide and the expression of transformation enzymes tapers very rapidly when they are cultured *in vitro*. Human hepatoma HepaRG cells are observed to express some CYPs, glutathione-S-transferases (GSTs) and UDP-glucuronosyl transferases (UGTs), after being isolated from a human hepatocellular cancer and cultured for two weeks [18]. Within several days after being thawed in culture the cells express several isoenzymes of CYPs, such as CYP3A4, 1A2, 2B6, 2C9 and 2D6 at levels comparable to primary human hepatocytes; however, CYP2E1 is absent. Correspondingly, the cells fail to activate styrene and ethanol, though strong micronuclei-induction and DNA breaks are observed with aflatoxin B1, cyclophosphamide and 7,12-dimethylbenzanthracene in these cells [19]. Moreover, the endogenous expression of biotransformation enzymes in these cells through continuing passages is not persistent, thus they are unsuitable for standard mutagenicity assays.

A possible remedy for these problems is the gene-technical expression of xenobiotic-metabolizing enzymes in cell lines commonly used in genotoxicity assays, although this approach is only realistic when sufficient information on the metabolism of the test compound is available, or structural alerts point to specific possible activation pathways. It is possible to express human enzymes, which offers an additional benefit as compared to rodent S9 preparations.

Recently we have observed that 1-methylpyrene (1-MP) and 1-hydroxymethylpyrene (1-HMP) are capable of inducing mutagenic responses in a Chinese hamster V79-derived cell line expressing both human CYP2E1 and human SULT1A1 (V79-hCYP2E1-hSULT1A1) [20], and also in this cell line we have observed activation of hydroquinone (HQ), catechol (CAT) and 1,2,4-trihydroxybenzene (THB) by human CYP2E1 to more genotoxic metabolites [21]. Of course, biotransformation reactions require time. Thus, exposure to the reactive metabolites formed in the cells occurs with some delay, as compared to the exposure to the parent test compound. Therefore, we were interested in whether the recommended treatment schedules mentioned above still fit for the *in vitro* micronucleus test with promutagens in V79-hCYP2E1-hSULT1A1 cells.

In this study, the effect of exposure/recovery schedule on micronuclei induction by known clastogens and aneugens was investigated in Chinese hamster V79 cells; similar studies were then conducted with 1-MP, 1-HMP, HQ, CAT and THB in V79-hCYP2E1-hSULT1A1 cells; lastly, the effects of the above chemicals on the mitotic index of these cells were determined, for an estimation of the presence of mitotic arrest, which may indicate depolymerization of microtubules, with some relevance to spindle aberrations and aneuploidy [22].

2. Materials and methods

2.1 Chemicals

HQ (CAS 123-31-9), CAT (CAS 120-80-9), THB (CAS 533-73-3), 1-MP (1-MP, CAS 2381-21-7) and colchicine (COL, CAS 64-86-8) were purchased from Sigma-Aldrich Co. (St. Louis, Mo); 1-HMP (CAS 24463-15-8) was from Tokyo Chemical Industry (Tokyo, Japan), mitomycin C (MMC, CAS 50-07-7) from Genview Scientific Inc. (Shanghai, China), bleomycin sulfate (BLM, CAS 9041-93-4) and vinblastine sulfate (VBL, CAS 143-67-9) from Medchem Express (Monmouth Junction, NJ). Pentachlorophenol (CAS 87-86-5) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). 1-MP, 1-HMP and pentachlorophenol were dissolved in dimethylsulfoxide (DMSO), and the other test chemicals were dissolved in the complete culture medium before exposing the cells.

2.2 Cell lines and culture

The V79 cell line was purchased from Shanghai Fuxiang Biotech (Shanghai, China). The V79-hCYP2E1-hSULT1A1 cell line was constructed by two steps of gene transfection: at first the wild-type human CYP2E1 was introduced by genetic engineering into V79-Mz cells leading to the production of cell line V79-hCYP2E1 [23], then the wild-type human SULT1A1 was introduced into V79-hCYP2E1 cells, and the cells from a selected colony stably expressing both enzymes were defined as V79-hCYP2E1-hSULT1A1 cell line [24]. Cells were cultured in Dulbecco's modification of Eagle's medium (Gibco, Shanghai, China) 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% CO₂.

2.3 Cytotoxicity test

The cytotoxicity of each chemical was determined using the CCK-8 assay (disulfonated tetrazolium salt serving as a chromogenic indicator for NADH and cell viability) according to a published method [20, 25]. Cells of each line were inoculated on 96-well plates at a density of 6.8×10^3 cells/well in 100 µL of culture medium. At 24 h, each test chemical was added at varying concentrations (MMC, BLM, COL and VBL to V79 cells; 1-MP, 1-HMP, HQ, CAT and THB to V79-hCYP2E1-hSULT1A1 cells). Change of medium was performed at varying time points, and cell cultures were terminated for CCK-8 assay uniformly at 48 h, so that a set of exposure/recovery schedules as 3 h/21 h, 6 h/18 h, 12 h/12 h, 18 h/6 h, and 24 h/0 h were attained. Six replicates were set up in each group. At 48 h, each culture was subject to CCK-8 treatment for 2 h, and finally the optical density of each sample at 450 nm (OD₄₅₀) was measured on a microplate reader (BioRad Model 680). Occasionally, for an extended (48 h) exposure of test compounds, cell density upon inoculation was

scaled down to 1.8×10^3 cells per well. As the solvent for 1-MP, 1-HMP or pentachlorophenol (10 μ M) (coexposure with HQ, CAT or THB, from 22 h to 48 h, for selective inhibition of SULT1A1, a detoxifying enzyme for these compounds) [21], DMSO was limited to a final concentration of 0.1% (v:v).

2.4 Micronucleus test

Two concentrations of each test chemical with no or mild cytotoxicity accompanied by obvious micronuclei induction, according to pretest results (with MMC, BLM, COL and VBL) or our recently published results (with the other test compounds) [20,21], were chosen for the following micronucleus test. The micronucleus test was conducted as previously described [20,21] with minor modification. Briefly, cells of each line were inoculated at the density of 3×10^5 to each 12.5-cm² flask containing 3 mL medium. At 24 h, cells were exposed to each test chemical at varying concentrations, with MMC, BLM, COL and VBL in V79 cells, and 1-MP, 1-HMP, HQ, CAT and THB in V79-hCYP2E1-hSULT1A1 cells. DMSO, used as the solvent of 1-MP and 1-HMP, was limited to the final concentration of 0.1% (v:v); complete culture medium was used to dissolve other chemicals. Duplicate cultures were set up for each treatment. Chemical exposure was terminated by a change of the medium at varying time points, and at 48 h cell cultures were terminated, so that various exposure/recovery schedules were set up (as described in section 2.3). For some test compounds that induced micronuclei with relatively long exposure duration and demonstrated cell survival greater than 50% after extended (48 h) exposure (by CCK-8 assay), micronucleus test with 48 h treatment/0 h recovery schedule was performed, with the cell density upon inoculation scaled down to 8×10^4 per flask. For the other test compounds (that started to induce micronuclei from short treatment/long recovery), further experiments were performed with a fixed treatment duration (12 h) and varying recovery periods (6, 12 and 18 h), with unchanged cell density upon inoculation. Pentachlorophenol (10 μ M) was present in the experiments with HQ, CAT, or THB in V79-hCYP2E1-hSULT1A1 cells, as described in section 2.3. Cells in each culture were harvested by trypsinization, subjected to hypotonic treatment, fixed in methanol:acetic acid (3:1) mixture, dropped onto glass slides, and finally stained with Giemsa. Under microscopy with the oil immersion lens (magnification of 1000), 1000 randomly encountered cells with integrate cell structure (cells with morphology of apoptosis or nucleus fragmentation were excluded) from each culture were scored for the frequency of micronucleated cells, each containing one or more micronuclei in the cytoplasm (separated from the main nucleus), by an experienced experimenter.

2.5 Mitotic index determination

The mitotic index was measured as previously described [26] with minor modification. A 4.5-cm² round

glass slide was placed onto each well (9.6 cm², 3 mL medium) of the 6-well culture boards; then, cells were inoculated at the density of 2×10^4 / cm² onto each well. At 24 h, cells in each culture were treated with each test chemical at two different concentrations (the same as in the micronucleus test). In case of V79-hCYP2E1-hSULT1A1 cells treated with HQ, CAT or THB, pentachlorophenol was present as described above for inhibition of SULT1A1. At 48 h, the culture medium was removed, and PBS was used to clean each well for twice. Immediately after each well got dry, cells were fixed with 1 mL of methanol:acetic acid (3:1) for 20 min. Then, the fix liquid was exchanged for a second 20 min fixation. When the wells were sufficiently dry, cells were stained with Giemsa. The glass slide in each culture was taken out for microscopic analysis; under 1000-fold magnification, 1000 randomly encountered and structurally integrate cells were scored for the frequency of mitotic cells.

2.6 Statistical analysis

ANOVA was performed to analyze the results of cytotoxicity assay with each test chemical, with the relative optical density (in percentage) in each treatment compared with the control group. For the micronucleus and mitotic index assays, data from duplicate experiments were combined to become quantal data, and then analyzed with the χ^2 test.

3. Results

3.1 Cytotoxicity and micronuclei induction by known clastogens and aneugens with varying treatment/recovery schedules in V79 cell line

As shown in Fig. 1 (panel A and E), MMC at 0.15 and 0.3 μ M did not change the cell growth/viability of V79 cells at exposures for 3–12 h, and only in the extended exposure conditions (≥ 18 h) marginal cytotoxicity was present. Similarly, as shown in Fig. 1 (B and F), BLM at 1 and 3 μ M showed no cytotoxicity in V79 cells at exposures for 3–6 h, and mildly cytotoxic under some of other schedules. However, as shown in Fig. 2 (panel A, B, C, and D), COL (0.15 and 0.3 μ M) and VBL (2 and 4 nM) were cytotoxic with all treatment schedules in V79 cells, with greater efficacy than MMC and BLM; noticeably, the effects of COL and VBL were treatment duration-dependent, both in the absence and presence of a recovery duration.

As shown in Fig. 1 (panel C and D), induction of micronuclei by both MMC and BLM in V79 cells displayed a biphasic feature: an exposure duration-dependent increase in micronuclei formation up to 12 h/12 h treatment, followed by a reduced effect with extended exposure durations; greater response at the higher concentration than at the low concentration were present with most schedules. With the treatment duration fixed at 12 h, recovery duration of 12 h and of 18 h gave rise to the greatest micronuclei induction by MMC

and BLM, respectively, as shown in Fig. 1 (panel G and H). Conversely, as shown in Fig. 2 (panel E and F), COL- and VBL-induced micronuclei formation elevated consistently along with the increase in exposure duration and decrease in recovery length, with a peaked response at 24 h/0 h; the response at the high concentration was stronger than at the low concentration. Apoptosis and nucleus fragmentation were occasionally encountered under microscopy at the high concentration of COL and VBL, and they were regarded as invalid cells excluded from scoring of micronucleated cells.

3.2 Cytotoxicity and micronuclei induction by 1-MP and 1-HMP in V79 and V79-hCYP2E1-hSULT1A1 cells

As shown in Fig. 3 (panel A and B), in V79-hCYP2E1-hSULT1A1 cells, 1-MP (10 and 20 μ M) and 1-HMP (2.5 and 5 μ M) induced mild to moderate or severe cytotoxicity under various exposure durations (without recovery), and 1-HMP caused a little stronger response with clearer exposure duration-dependence than 1-MP. As shown in Fig. 3 (panel C and D), with a recovery period they demonstrated cytotoxicity at levels similar to that without recovery (described above). As shown in Fig. 3 (panel E and F), both 1-MP and 1-HMP elevated the frequency of micronucleated cells along with the increase in exposure duration, peaked at 18 h/6 h, followed by a decrease at the 24 h/0 h treatment; there was a stronger response at the high concentration than the low concentration consistently over various treatment schedules.

In order to ascertain the role of CYP2E1/SULT1A1 in activating 1-MP and 1-HMP, induction of micronuclei by 1-MP and 1-HMP in both V79 and V79-hCYP2E1-hSULT1A1 cells with 12 h/12 h and 24 h/0 h treatment schedules was determined. As indicated in Table 1, with the 12 h/12 h schedule, neither 1-MP nor 1-HMP at the indicated concentrations induced micronuclei, however, they both significantly elevated the frequency of micronucleated cells in V79-hCYP2E1-hSULT1A1 cells. With the 24 h/0 h schedule, 1-HMP was inactive toward V79 cells, while it induced micronuclei in V79-hCYP2E1-hSULT1A1 obviously; 1-MP at the low concentration (10 μ M) did not induce micronuclei in V79 cells, but it elevated the frequency of micronucleated cells in V79-hCYP2E1-hSULT1A1 cells by several times with statistical significance; 1-MP at the higher concentration (20 μ M) induced micronuclei in both cell lines, however, the response in V79-hCYP2E1-hSULT1A1 cells was significantly greater than in V79 cells.

3.3 Cytotoxicity and micronucleation by HQ, CAT and THB in V79 and V79-hCYP2E1-hSULT1A1 cell lines

To ascertain whether or how much CYP2E1 might contribute to the activity of HQ, CAT, and THB to induce micronuclei in V79-hCYP2E1-hSULT1A1 cells, cytotoxicity and micronucleus test were performed with each compound in both V79 and V79-hCYP2E1-hSULT1A1 cell line (in the latter cell line 10 μ M of PCP was present for inhibition of SULT1A1), with a 12 h/12 h schedule. As indicated in Table 2, each compound

was mildly cytotoxic in both cell lines (no obvious difference between effects in the cell lines). Somehow the induction of micronuclei by HQ, CAT and THB in V79 cells was weaker than that in a previous report [27], however, micronuclei induction by each compound in V79-hCYP2E1-hSULT1A1 cells was significantly stronger than in V79 cells. This evidenced that CYP2E1 expressed in V79-hCYP2E1-hSULT1A1 cells may have activated HQ, CAT, and THB to more mutagenic metabolites.

As shown in Fig. 4 (panel A, B, and C), HQ, CAT and THB showed marginal cytotoxicity under most treatment/recovery schedules, and no effect under other schedules. For micronuclei induction each of HQ, CAT and THB demonstrated biphasic features, with the effect of HQ peaking at the 12 h/12 h and 18 h/6 h schedules, and that of CAT and THB at the 6 h/12 h schedule, as shown in Fig. 4 (panel D, E, and F). Under experiment schedules with fixed treatment duration (12 h) and varying recovery length (6, 12 and 18 h), the cytotoxicity of each test compound was unchanged or mildly varied; the relatively optimal schedule for micronuclei induction by HQ and CAT was with recovery duration of 12 h, and that by THB seemed to be both 12 h and 6 h (Fig. 4G, H, and I). Stronger micronuclei induction was consistently observed at the high concentration with varying treatment schedules than at the low concentration.

3.4 Induction of mitotic arrest by each test compound in V79 and V79-hCYP2E1-hSULT1A1 cell lines

As shown in Fig. 5A, MMC and BLM had no effect on the mitotic index of V79 cells, while COL and VBL significantly elevated the mitotic index. Interestingly, the mitotic indices of V79 cells with COL and VBL at higher concentrations were lower than that at the lower concentrations. Since the growth of V79 cells was not enhanced by each chemical (actually inhibited by COL and VBL, as shown in Fig. 1), an increase in the mitotic index was indicative of mitotic arrest. As shown in Fig. 5B, HQ and CAT did not change the mitotic index of V79-hCYP2E1-hSULT1A1 cells, and THB (at the lower concentration) marginally elevated it; on the contrary, 1-MP and 1-HMP apparently elevated the mitotic index, and the values at the higher concentrations were greater than that at the lower concentrations. We also checked the effects of 1-HMP and 1-MP on the frequency of mitosis in V79 cells after 24 h exposure, and the results revealed a value of 14 ± 1 (‰) as the control, 16 ± 0 and 15 ± 5 (‰) with 1-HMP at the concentration of 2.5 μ M and 5 μ M respectively, and 22 ± 1 and 27 ± 4 (‰) with 1-MP at the concentration of 10 μ M and 20 μ M respectively. The only statistical significance ($p < 0.01$, by χ^2 test after combining the duplicate data) in comparison with the control was observed in the group with 20 μ M of 1-MP, with a two-fold increase in the frequency of mitosis. These findings, along with the observed reduction of cell growth/viability, indicate that 1-MP and 1-HMP actively induced mitotic arrest in V79-hCYP2E1-hSULT1A1 cells, though a mild effect of 1-MP at high concentrations

in V79 cells could be present.

4. Discussion

The two concentrations of each test chemical applied in this study were associated with statistically significant elevation of the frequency of micronucleated cells under several treatment schedules over the control, accompanied with no or mild to moderate cytotoxicity. As such, MMC and BLM showed no or just marginal cytotoxicity in V79 cells; however, COL and VBL induced mild to moderate cytotoxic effects in an exposure-time-dependent manner. This may be related to the mitotic arrest induced by COL and VBL, as was evidenced by the increased mitotic index in V79 cells, which is consistent with previous findings that COL and VBL are both mitotic arrest inducers [28]; indeed, accumulation of mitotic cells due to incomplete mitosis may lead to reduced cell growth. Meanwhile, the absence of substantial cytotoxicity of MMC and BLM at concentrations just above their genotoxic thresholds in V79 cells seems accordant to the unchanged mitotic frequencies in V79 cells. More obviously, the induction of micronuclei by known clastogens (MMC and BLM) and known aneugens (COL and VBL) in V79 cells demonstrate significantly varied intensities of action under varied treatment/recovery durations, with the effects of clastogens and aneugens peaking at different schedules, i.e., 12 h/12 h and 24 h/0 h, respectively. MMC is a cross-linking agent, after reductive activation it may damage DNA by alkylating guanine residues, leading to DNA adducts and DNA interstrand and intrastrand crosslinks [29]. BLM exerts its genotoxicity by generating free radicals, which attack C-4' in the deoxyribose backbone of DNA, leading to opening of the ribose ring and strand breakage; it is an S-independent radiomimetic agent that causes double-strand breaks in DNA [30]. Both MMC and BLM are typical clastogens, while COL and VBL are typical aneugens targeting on the intracellular microtubule system and causing chromosome loss during mitosis [31]. Our results with these mutagens of known modes of action are in accordance to the statement outlined in the OECD guidelines for the *in vitro* micronucleus test that a short exposure/long recovery schedule is favorable for detection of clastogens while a long exposure/short recovery schedule facilitates micronuclei induction by aneugens. Studies with known clastogens and aneugens in other cell models also provide similar results. For example, when diethylstilbestrol (DES, an aneugen) [32] and 4-nitroquinoline-*N*-oxide (4-NQO, a clastogen) [32] were both tested for induction of micronuclei in primary cultures of fibroblasts derived from ram seminal vesicles (whose doubling time is about 16 h), it was observed that 4-NQO and DES showed maximal micronuclei induction after 24 h and 12 h of recovery, respectively [33]. Similar results with these two mutagens in primary cultures of Syrian hamster embryo and ovine seminal vesicle cells were also obtained [32]. Furthermore, our findings about varied treatment/recovery schedules

favorable for micronuclei induction by clastogens and aneugens may not cover all mutagens unexceptionally, since in some cases such as the presence of cell growth retardation, the kinetics of micronuclei induction may also be delayed to some extent. Therefore, it requires the use of suitable molecular techniques such as fluorescent anti-kinetochore staining and fluorescent in situ hybridization (FISH) to identify mutagens as clastogens and aneugens, rather than merely according to the maximal response-related treatment schedule.

In this study, two sets of other chemicals, i.e., polyhydroxylated metabolites of benzene (HQ, CAT and THB) and alkylated pyrene compounds (1-MP and 1-HMP), were investigated for the induction of micronuclei in V79-hCYP2E1-hSULT1A1 cells with varying exposure/recovery schedules. 1-MP may be converted to a reactive intermediate through two metabolic steps, i.e., benzylic hydroxylation by a CYP enzyme and subsequent sulfo-conjugation of the hydroxyl group by a SULT enzyme [35,36]; the other compounds need one step of biotransformation, i.e., sulfo conjugation of 1-HMP by a SULT enzyme [36] and oxidation of HQ, CAT and THB by CYP2E1 [21]. As observed in this study, CAT and THB induced micronuclei in V79-hCYP2E1-hSULT1A1 cells with peak effects under the 6 h/18 h treatment schedule, exposure duration even slightly shorter than that of the known clastogens (12 h/ 12h). This result suggests that the kinetics of metabolic activation of CAT and THB by human CYP2E1 may not be an issue to delay micronuclei formation to a relevant extent under the conditions used. Additionally, 1-MP and 1-HMP, which require either two- or one-step of metabolically activating reaction for the formation of a reactive intermediate, demonstrated almost identical treatment schedule-micronuclei induction pattern in V79-hCYP2E1-hSULT1A1 cells; this pattern was relatively close to that of VBL and COL. The relevance of the observed effects of 1-HMP and 1-MP to the heterologously expressed enzymes in V79-hCYP2E1-hSULT1A1 cells is evidenced by the presence of micronuclei induction by 1-HMP at both test concentrations and 1-MP at the lower concentration with varied treatment schedules in V79-hCYP2E1-hSULT1A1 cells and the negative results in V79 cells (as indicated in table1). The elevation of the frequency of micronucleated cells in V79 cells after treatment with 1-MP at 20 μ M for 24 h, though significantly lower than the value in V79-hCYP2E1-hSULT1A1 cells, may represent a biotransformation enzyme-independent activity of 1-MP toward V79 cells. Taken together, the above evidence indicates that the biotransformation reactions required for the genotoxicity of these chemicals may not change the relationship between the efficiency of micronuclei induction and the treatment schedule; in other words, the standard exposure/recovery schedules for micronucleus test *in vitro* may still be applicable with promutagens as test compounds in biotransformation enzyme-expressing cells. This remains to be verified for other compounds, as biotransformation rates and levels of metabolites required for a genotoxic effect may vary

between different compounds.

In this study, 1-MP and 1-HMP require long exposure/short recovery as preferred schedule while a different schedule is favorable for the effects of CAT and THB. Moreover, these two categories of chemicals are opposite to each other in their ability to induce mitotic arrest. This may present a new example for the chemical structure-biological activity relevance. Moreover, since mitotic arrest is associated with spindle disturbance and aneuploidy, and most mitotic arrestants are also aneugens, such as COL, VBL, naturally occurring and synthetic estrogens (e.g., DES), and various bisphenols (e.g., bisphenol A), etc. [37–40], the induction of mitotic arrest by 1-MP and 1-HMP in V79-hCYP2E1-hSULT1A1 cells in this study is also an indirect evidence suggesting a possible aneugenic effect. Here, the relevance of the induction of mitotic arrest in V79-hCYP2E1-hSULT1A1 cells by 1-HMP and 1-MP to the enzymes heterologously expressed in the cells is supported by the absence of a statistically significant elevation of mitotic frequency with 1-HMP and 1-MP (the latter compound at the lower concentration), and with 1-MP at the high test concentration the elevation of the frequency of mitosis in V79-hCYP2E1-hSULT1A1 cells is substantially greater than in V79 cells. After all, the possibility of 1-MP and 1-HMP as aneugens needs to be confirmed with molecular techniques to visualize kinetochore-positive micronuclei. It is true that up to now no identified aneugens require metabolic activation, however, the limited exposure duration (no longer than 6 h) with S9-mix as a standard *in vitro* bioactivating system for the micronucleus test, which is unfavorable for detection of aneugens, may have decreased the sensitivity of tests to identify promutagens, including aneugenic promutagens.

HQ, CAT and THB may individually or synergistically form DNA adducts in human cells as well as cell-free medium [41,42]. Moreover, HQ may induce frequent base deletions and point mutations in human cells using an *supF* forward mutation assay [43]. HQ has been observed to be both aneugenic and clastogenic in human blood lymphocytes [44] and V79 cells [45], even though the metabolizing activity associated with CYPs in these cells is absent or very limited, unlike myeloperoxidase, which may mediate the activation of benzene metabolites in these cells [46,47]. THB has also been observed to induce aneuploidy in cultured human lymphocytes using a FISH technique [48]. Moreover, CAT, HQ and THB may promote the generation of reactive oxygen species [49], thus contribute to DNA breaks. Lastly, in our cell model they may be further activated to more genotoxic metabolites by CYP2E1. Therefore, the factors influencing the time course of micronuclei induction in the cells are multiple and complex. This complexity is reflected by the time courses of micronuclei formation under 12 h exposure and varying durations of recovery, which do not resemble typical clastogens or aneugens, but range between them (Fig. 4J, K and L).

In summary, this study suggests that the time course of micronuclei induction by the test promutagens in V79-hCYP2E1-hSULT1A1 cells may not be restricted by the enzyme-catalyzed activating reactions. Furthermore, 1-MP and 1-HMP induce micronuclei in these cells with preferred treatment schedules different from the polyhydroxylated metabolites of benzene under intracellular bioactivation, where 1-MP and 1-HMP attain a mitotic arresting activity.

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Table 1 Induction of micronuclei in V79 and V79-hCYP2E1-hSULT1A1 cells by 1-methylpyrene and 1-hydroxymethylpyrene

Chemicals	μM	Frequency of micronucleated cells, ‰			
		12 h/12 h schedule		24 h/0 h schedule	
		V79 cells	V79-hCYP2E1-hSULT1A1	V79 cells	V79-hCYP2E1-hSULT1A1
Control		9 $\pm$ 1	6 $\pm$ 1	7 $\pm$ 1	10 $\pm$ 1
1-MP					
	10	14 $\pm$ 2	36 $\pm$ 4** ^{###}	12 $\pm$ 1	45 $\pm$ 2** ^{###}
	20	14 $\pm$ 1	66 $\pm$ 7** ^{###}	36 $\pm$ 2**	78 $\pm$ 14** ^{###}
1-HMP					
	2.5	12 $\pm$ 1	23 $\pm$ 2** ^{###}	11 $\pm$ 1	28 $\pm$ 2** ^{###}
	5	13 $\pm$ 2	90 $\pm$ 4** ^{###}	7 $\pm$ 1	114 $\pm$ 20** ^{###}

Cells were treated with 1-methylpyrene (1-MP) and 1-hydroxymethylpyrene (1-HMP) at the indicated concentrations under the treatment/recovery schedules of 12 h/12 h or 24 h/0 h. The frequency of micronucleated cells in each group was expressed as means and range of variation (n = 2), and statistically analyzed with χ^2 examination after combining the duplicate data. ** p < 0.01, compared with the control; ^{###} p < 0.01, a comparison was made between experiments in V79 and V79-hCYP2E1-hSULT1A1 cells.

Table 2 Cytotoxic and micronuclei-inducing activities of hydroquinone, catechol, and 1,2,4-trihydroxybenzene in V79 cells and V79-hCYP2E1-hSULT1A1 cells

Test chemicals	Concentration (μM)	Cell survival, %		Micronucleated cells, ‰	
		V79 cells	V79-hCYP2E1-hSULT1A1 cells	V79 cells	V79-hCYP2E1-hSULT1A1 cells
None		100 ± 3	100 ± 3	6 ± 2	8 ± 1
HQ					
	5	90 ± 7**	93 ± 3**	11 ± 1	15 ± 2*
	10	74 ± 7**	75 ± 5**	20 ± 0**	62 ± 10*** ^{##}
CAT					
	10	101 ± 7	93 ± 3** [#]	11 ± 1	16 ± 3*
	20	89 ± 5**	92 ± 4**	12 ± 2*	23 ± 0*** ^{##}
THB					
	30	96 ± 7	90 ± 4**	10 ± 2	17 ± 2*** [#]
	60	89 ± 10**	88 ± 5**	23 ± 2**	50 ± 1*** ^{##}

Cells were treated with hydroquinone (HQ), catechol (CAT), or 1,2,4-trihydroxybenzene (THB) for 12 h, followed by test chemical-free culturing (recovery) for another 12 h. To inhibit the SULT1A1 activity in V79-hCYP2E1-hSULT1A1 cells, pentachlorophenol (10 μM) was present from 2 h prior to addition of test chemicals to the end of cell recovery. Cell growth/viability was assay by the CCK-8 method, with the results expressed as means and S.D. (n = 6), and statistical analysis performed using ANOVA. The frequency of micronucleated cells in each group was expressed as means and range of variation (n = 2), and statistically analyzed with χ^2 examination after combining the duplicate data. * p < 0.05, ** p < 0.01, compared with the control; [#] p < 0.05, ^{##} p < 0.01, comparison was made between experiments in V79 and V79-hCYP2E1-hSULT1A1 cells.

Figure legends

Fig. 1 Cytotoxicity and micronuclei induction by mitomycin C and bleomycin in Chinese hamster V79 cell line with varying exposure/recovery schedules

Cells were exposed to each chemical at two different concentrations with the following exposure/recovery schedules: (1) 0 h/24 h, 3 h/21 h, 6 h/18 h, 12 h/12 h, 18 h/6 h and 24 h/0 h, with the combination of treatment and recovery durations fixed as 24 h (panel A, B, C, and D); (2) treatment duration fixed at 12 h, with varying recovery durations (6, 12, and 18 h) (panel E, F, G and H). Cytotoxicity was measured by the CCK-8 assay, with results expressed as the optical density at 450 nm. Data of the CCK-8 assays are means $\pm$ SD of six replicates. Data of the micronucleus test are means $\pm$ half ranges of variation of two replicates; however, the duplicate data are combined to become quantal data for nonparametric statistical analysis. * $p < 0.05$, ** $p < 0.01$, compared with the control by an ANOVA; [#] $p < 0.05$, ^{##} $p < 0.01$, compared with the control by a χ^2 test.

Fig. 2 Cytotoxicity and micronuclei induction by colchicine and vinblastine in Chinese hamster V79 cell line with varying exposure/recovery schedules.

See the legend of Fig. 1. Cytotoxicity of each test compound was determined with varying treatment periods (3, 6, 12, 18, 24, and 48 h) and zero recovery (panel A and B), or with varying treatment/recovery combinations (total periods fixed as 24 h) (panel C and D). The micronucleus test was performed with the latter set of treatment/recovery schedules. * $p < 0.05$, ** $p < 0.01$, compared with the control by an ANOVA; [#] $p < 0.05$, ^{##} $p < 0.01$, compared with the control by a χ^2 test.

Fig. 3 Cytotoxicity and micronuclei induction by 1-methylpyrene and 1-hydroxymethylpyrene in V79-hCYP2E1-hSULT1A1 cells with varying exposure/recovery schedules.

See the legend of Fig. 1.

Fig. 4 Cytotoxicity and micronuclei induction by hydroquinone, catechol and 1,2,4-trihydroxybenzene in V79-hCYP2E1-hSULT1A1 cells with varying exposure/recovery schedules.

See the caption of Fig. 2. Cells were coexposed to pentachlorophenol (10 μ M) for selective and complete inhibition of SULT1A1 activity, which would otherwise conjugate and thus inactivate the test compounds, from 22 h to 48 h [21].

Fig. 5 Effects of hydroquinone, catechol, 1,2,4-trihydroxybenzene, 1-methylpyrene and 1-hydroxymethylpyrene on the frequency of mitosis in V79-hCYP2E1-hSULT1A1 cells.

Cells were exposed to each test chemical for 24 h (without recovery), then subjected to fixation and Giemsa staining. Mitomycin C, bleomycin, colchicine and vinblastine were used as known clastogens and aneugens (positive controls) in V79 cells (panel A). The concentrations of each chemical were consistent with those applied in the cytotoxicity and micronucleus tests (shown in Fig. 1–4). Under microscopy, 1000 randomly encountered cells in each culture were scored for the frequency of mitotic cells. Data are means and ranges of variation of two replicates. For a χ^2 test, the duplicate data are combined to become quantal data; * $p < 0.05$, ** $p < 0.01$, compared with the control.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5