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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 the 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.

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

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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 are favorable for detection of aneugen-induced micronuclei, while a short exposure and extended recovery are 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 sulfoconjugate 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) was 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% CO_2.