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Development and validation of an in vitro micronucleus assay platform in TK6 cells

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ABSTRACT

The Organization for Economic Co-operation and Development (OECD) has recently adopted Test Guideline 487 (TG487) for conducting the in vitro micronucleus (MNvit) assay. The purpose of this study is to evaluate and validate treatment conditions for the use of p53 competent TK6 human lymphoblastoid cells in a TG487 compliant MNvit assay. The ten reference compounds suggested in TG487 (mitomycin C, cytosine arabinoside, cyclophosphamide, benzo-*a*-pyrene, vinblastine sulphate, colchicine, sodium chloride, nalidixic acid and di(2-ethylhexyl)phthalate and pyrene) and noscapine hydrochloride were chosen for this study. In order to optimize the micronucleus response after treatment with some positive substances, we extended the recovery time after pulse treatment from 2 cell cycles recommended in TG487 to 3 cell cycles for untreated cells (40 h). Each compound was tested in at least one of four exposure conditions: a 4 h exposure followed by a 40 h recovery, a 4 h exposure followed by a 24 h recovery, a 4 h exposure in the presence of an exogenous metabolic activation system followed by a 40 h recovery period, and a 27 h continuous direct treatment. Results show that the direct acting clastogens, clastogens requiring metabolic activation and aneugens caused a robust increase in micronuclei in at least one test condition whereas the negative compounds did not induce micronuclei. The negative control cultures exhibited reproducibly low and consistent micronucleus frequencies ranging from 0.4 to 1.8% (0.8 ± 0.3% average and standard deviation). Furthermore, extending the recovery period from 24 h to 40 h produced a 2-fold higher micronucleus frequency after a 4 h pulse treatment with mitomycin C. In summary, the protocol described in this study in TK6 cells produced the expected result with model compounds and should be suitable for performing the MNvit assay in accordance with guideline TG487.

1. Introduction

Micronuclei form when chromosome fragments or whole chromosomes remain in the cytoplasm after cell division. The in vitro micronucleus assay has been extensively used for decades to identify clastogenic and aneugenic agents. However, the Organization for Economic Co-operation and Development (OECD) has only recently adopted test guideline TG487 for conducting the MNvit assay in order to harmonize the conduct of the assay across laboratories [1]. Acceptability criteria outlined in the OECD guidance state that a cell line is suitable for performing the MNvit assay when it is demonstrated to reliably and accurately detect substances of known aneugenic and clastogenic activity. Additionally, the cells should exhibit reproducibly and consistently low negative control micronucleus frequencies.

In 2006, the European Centre for the Validation of Alternative Methods (ECVAM) held a two-day workshop to address the high rate of misleading in vitro mammalian assay positive findings, i.e. compounds that are positive in the in vitro mammalian chromosomal damage assays but do not produce positive results in the in vivo genotoxicity assays or in rodent carcinogenicity testing. One promising solution for reducing the rate of misleading positive results proposed by the workshop participants was to use human cell lines that are karyotypically stable, p53 proficient, and DNA repair proficient [2–4]. The TK6 human B lymphoblastoid cell line is p53-competent, karyotypically stable and produces fewer misleading positive results than p53-mutated rodent cell lines [4]. The spontaneous frequency of mutations and chromosome alterations in these cells are not different from primary human cells [5]. Furthermore, TK6 cells show an 80–90% concordance with primary human lymphocytes in both the micronucleus assay and the

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chromosome aberration assay in a study of 80 compounds [3]. The advantage of using TK6 cells is that it is a well-studied human cell line and its use eliminates the donor to donor variability observed with primary human lymphocytes.

Evidence exists in literature that TK6 cells have prolonged cell cycle inhibition in response to DNA damage. Specifically, potent and direct acting mutagens such as mitomycin C and methyl methanesulfonate cause substantial cell cycle arrest 20 h after pulse treatment with approximately 60% of cells arrested in G_2/M [6]. Previous studies have shown that TK6 cells require an extended recovery time after a pulse treatment in order to optimize micronucleus expression [7,8]. Therefore, the recovery time after a pulse treatment used in the current validation study was extended from 2 cell cycles (recommended in OECD guidance) to 3 cell cycles for untreated cells.

The goal of the current study is to evaluate and improve sensitivity of the micronucleus assay in TK6 cells in accordance with OECD guideline TG487. Ten reference compounds suggested in TG487 (mitomycin C, cytosine arabinoside, cyclophosphamide, benzo-*a*-pyrene, vinblastine sulphate, colchicine, sodium chloride, nalidixic acid and di(2-ethylhexyl)phthalate and pyrene) and noscapine hydrochloride were chosen for this study. Each compound was tested in at least one of four exposure conditions: a 4 h direct exposure followed by a 40 h recovery period (4/40), a 4 h direct exposure followed by 24 h recovery period (4/24), a 4 h exposure in the presence of an exogenous metabolic activation system followed by a 40 h recovery period (4/40 + S9) and a 27 h continuous direct treatment.

2. Materials and methods

2.1. Cell culture

Frozen cell stocks were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and stored at −80 °C. Cultured cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere. Cells were cultured in RPMI 1640 + L-glutamine media (Lonza, Walkersville, MD) supplemented with heat-inactivated fetal bovine serum (FBS) (Mediatech, Manassas, VA) and penicillin–streptomycin (Invitrogen, Grand Island, NY). Stock cultures were passed 2–4 times per week at an initial density between 0.01 and 0.2 × 10^5 cells/mL and maintained such that the culture density did not exceed 7 × 10^5 cells/mL at time of passage. During the course of the validation studies, the average population doubling time for TK6 cells was 13 (±1.32) h:

$$\text{Population doubling time (PDT)} = \frac{1}{[([\log(\text{cell density}^b/\text{cell density}^a)]/\log 2)/\text{elapsed time}]}$$

where b = current cell density and a = previous cell density.

2.2. Metabolic activation system

The in vitro metabolic activation system was comprised of a rat liver homogenate fraction prepared from male Sprague Dawley rats treated with Aroclor 1254 (S9; purchased from Moltox, Boone, NC) and an energy-source containing NADPH (Sigma Aldrich, St. Louis, MO, USA).

Immediately prior to test article exposure, the S9 homogenate was thawed at room temperature and placed on ice. The S9 homogenate was diluted in RPMI 1640 without addition of FBS and then combined with NADPH. The concentration of NADPH in the mix was 0.02 mg/mL and the concentration of S9 was 30% (volume/volume). The S9 mix was added to the cell suspension at a ratio of 2 mL per 100 mL cell suspension (0.6% S9 in treatment culture).

2.3. Toxicity characterization

Cytotoxicity was characterized as the relative population doubling of the treated cultures compared to the population doublings of the concurrent negative control cultures. It is calculated as follows:

$$\text{Population doubling (PD)} = \frac{[\log(\text{cell density}^b/\text{cell density}^a)]}{\log 2}$$

Relative population doubling (RPD) = (the number of population doublings in the treated cultures/the number of population doublings in the negative controls cultures) × 100

$$\%\,\text{Toxicity} = (100 - \text{RPD})$$

where b = current cell density and a = previous cell density.

An automated cell counter (Coulter Counter Z1 or ZM, Beckman Coulter) was used to determine cell culture density (lower threshold at 7 μm).

2.4. Test article preparation

The solvent of choice for the dilution and delivery of the test article was dimethyl sulfoxide (DMSO). If the test article did not dissolve in DMSO, then culture media was used. To minimize any effects of the solvent on the test system, the final concentration of DMSO in the culture media was limited to 1% (volume/volume). To achieve this, highly concentrated (100×) test article dosing solutions or suspensions were prepared. Duplicate cultures were treated with each test article at each concentration. Table 1 contains the compounds tested.

2.5. Micronucleus assay procedures

Approximately 24 h prior to treatment with test article, TK6 cell stock cultures were prepared in vented T-75 cm^2 flasks at a cell density of 1.0–1.8 × 10^5 cells/mL. Stock cultures were incubated at 37 °C in 5% CO_2 in upright flasks for 22–26 h.

Prior to utilizing cells for dosing, the population doubling (PD) was calculated to determine whether the cells were growing at an acceptable rate. An acceptable PD range for an approximately 24 h incubation period was between 1.2 and 2.0. The cell density of the stock cell suspension was adjusted to 2.5–3.5 × 10^5 cells/mL and 2.5 mL aliquots were dispensed into 15 mL centrifuge tubes (4 h pulse treatments) or 10–12 mL aliquots were dispensed into T-25 cm^2 tissue culture flasks (27 h continuous treatment).

2.5.1. Test article treatment

Generally, 12 concentrations of the test article were tested in duplicate under each treatment condition. Appropriate solvent vehicle controls were tested concurrently for each treatment condition. For the 4/40 and 4/24 tests with and without metabolic activation, doses were delivered by adding 25 μL of a 100× stock solution to 2.5 mL of cell suspension. For the 27 h direct test, doses were delivered by adding 10–12 μL of a 100× stock solution to 10–12 mL of cell suspension.

2.5.2. Wash

At the end of each 4 h treatment, culture tubes were centrifuged for 6 min at 1000 rpm, the supernatant was removed, and the cell pellets were resuspended in residual supernatant by agitation of tubes. Next, 10 mL of media was added to each culture (5 mL for the 4/24 condition) and cell suspensions were transferred to T-25 cm^2 tissue culture flasks. The cultures were then incubated for 20 h or 40 h.

2.5.3. Cell harvest

Aliquots were removed from each culture and cell densities calculated. Five milliliters of each cell culture was transferred to 15 mL centrifuge tubes followed by addition of 5 mL room temperature 75 mM potassium chloride hypotonic solution, tubes were inverted to mix. After an approximate 4 min incubation at room temperature, 1 mL of room temperature fixative solution (9:1 volume methanol:acetic acid) was layered on top of the hypotonic solution in each culture and the tubes were inverted to mix. Cultures were immediately centrifuged at 1000 rpm for 6 min. The supernatant was decanted and the cell pellets were resuspended in residual supernatant. Approximately 1 mL of fixative solution was added to each tube and inverted to mix. An additional 3–5 mL of fixative was added to all tubes and inverted to mix. Samples were stored at 1–8 °C until slide preparation.

2.6. Evaluation for micronuclei induction

At least three test concentrations and the concurrent vehicle control were selected for analysis from each test. The highest test concentration was determined on a case by case basis taking into account both solubility and relevant cytotoxicity information available on the test article. For most compounds the highest concentration produced an approximate 55 ± 5% toxicity. In the absence of insolubility or limiting cytotoxicity, 5000 (g/mL or 10 mM (whichever was lower) was used as the highest active concentration. For non-toxic, insoluble compounds, dosing schemes were designed to include at least one level that exhibited evidence of precipitate in the culture medium.

Cells were centrifuged at 1000 rpm for 6 min, resuspended in fresh 9:1 fixative solution, dropped onto glass slides and air dried. The slides were stained in a solution of Acridine Orange and PBS. Blind-coded slides were analyzed with an epi-fluorescence microscope under a high magnification (200–400×). At least 1000 mononucleated cells from each duplicate culture were analyzed for the presence of micronuclei (2000 mononucleated cells per concentration). Binucleated and multinucleated cells were not analyzed.