Regulatory Toxicology and Pharmacology 59 (2011) 28–36

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

# Comparison of four different treatment conditions of extended exposure in the *in vitro* micronucleus assay using TK6 lymphoblastoid cells

Kiyohiro Hashimoto *, Yumi Nakajima, Shigeo Matsumura, Fumio Chatani

*Development Research Center, Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, 17-85, Jusohonmachi 2-chome, Yodogawa-ku, Osaka 532-8686, Japan*

ARTICLE INFO

*Article history:*
Received 19 April 2010
Available online 25 August 2010

*Keywords:*
TK6 cells
Cytokinesis block
Recovery period
Treatment conditions for extended exposure

ABSTRACT

In the OECD Guideline 487, a total of four extended exposure treatment conditions are proposed for the *in vitro* micronucleus (MNvit) assay in the presence and absence of a cytokinesis block and with or without a recovery period. This guideline also states that rodent cell lines and human lymphocytes can be used as shown by many validated studies but that human cell lines such as TK6 and HepG2 are not yet validated. In this present study each extended exposure condition was characterized by investigation using TK6 cells and nine chemicals known to be able to induce micronucleus (MN) in rodent cell lines. The results revealed two concerns: six chemicals did not show significant MN induction in the 'cytokinesis block without recovery period'; two aneugens showed no dose-dependent cytotoxicity in the 'cytokinesis block with recovery period'. Further investigation revealed that 3–4 times higher spontaneous MN frequency than that in the other conditions is a possible reason for the low sensitivity, and this high spontaneous MN frequency was not observed in Chinese hamster lung cells under the identical treatment condition. With regard to the two conditions without cytokinesis block, two negative substances were evaluated and found to be negative, suggesting the validity of the TK6 test system for these conditions. Although our findings showed a few concerns for the treatment with cytokinesis block, the TK6 cells were considered to be a reliable cell line to be used for detecting potential inducers of MN in the *in vitro* micronucleus assay based on the overall results.



## 1. Introduction

The *in vitro* micronucleus (MNvit) assay has been widely used in genotoxicity testing, especially as a screening tool for genotoxicity evaluation in the development of pharmaceuticals. A standard protocol was designed at the International Workshops on Genotoxicity Testing (IWGT) (Kirsch-Volders et al., 2000,2003), and Organization for Economic Co-operation and Development (OECD) guideline is now available (OECD Guideline for the Testing of Chemicals, 2010. No. 487). Data from the assay have been accumulated through the literature (Garriott et al., 2002) and several collaborative validation studies (Lorge et al., 2006; Matsushima et al., 1999; von der Hude et al., 2000). Also, extensive studies have shown the utility of the MNvit assay for detecting clastogens and aneugens and have confirmed that various cell lines are suitable for scoring micronuclei (Aardema et al., 2006; Clare et al., 2006; Oliver et al., 2006; von der Hude et al., 2000; Wakata et al., 2006).

In the OECD Test Guideline 487, the rodent derived cell lines: CHO, CHL, V79, and L5178Y, and human peripheral blood lymphocytes are recommended as validated cells because extensive data on these cells are robust and reproducible (Aardema et al., 2006; Albertini et al., 1997; Clare et al., 2006; Garriott et al., 2002; Kalweit et al., 1999; Kersten et al., 1999; Lorge et al., 2006; Matsushima et al., 1999; Miller et al., 1997,1998; Oliver et al., 2006; von der Hude et al., 2000; Wakata et al., 2006). Human derived cell line TK6 is also included in the guideline as options; however, less data is available for the cell line so that the present study aimed to provide further data with TK6 cells. The latest IWGT, held in Switzerland in 2009 reached a conclusion that p53 competent cells are preferable to p53 compromised cell lines for the evaluation of the MN induction potential for human risk assessment, because of irrelevant positive results obtained in the p53 compromised rodent cell lines (www.iaems.net/IWGT/IWGT_Group3_better-predictive-alternatives.ppt). Around the same time as this statement, TK6, one of the human derived cell lines, attracted considerable

*Abbreviations:* MN, micronucleus; MNvit, *in vitro* micronucleus; IWGT, International Workshops on Genotoxicity Testing; OECD, Organisation for Economic Co-operation and Development; CHO, Chinese hamster ovary; CHL, Chinese hamster lung; MMS, methylmethanesulfonate; MMC, mitomycin C; BLM, bleomycin; Ara C, cytosine-1-β-D-arabinofuranoside; APC, aphidicolin; HU, hydroxyurea; COL, colcemid; BEN, benomyl; TBZ, thiabendazole; DMSO, dimethyl sulfoxide; NIHS, National Institute of Health Science; CyB, cytochalasin B; RP, recovery period; RPD, relative population doubling; RI, replicative index; SFTG, Société Française de Toxicologie Génétique; MFI, maximal fold increase.

* Corresponding author. Tel.: +81 6 6300 6469; fax: +81 6 6300 6918.
*E-mail address:* Hashimoto_Kiyohiro@takeda.co.jp (K. Hashimoto).

0273-2300/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.yrtph.2010.08.016

attention for genotoxicity testing (Bryce et al., 2007; Ellinger-Ziegelbauer et al., 2009; Fellows and O'Donovan, 2009; Platel et al., 2009).

The OECD Test Guideline 487 describes a detailed protocol for the MNvit assay, allowing several treatment conditions for extended exposure: an option of with or without the actin polymerization inhibitor cytochalasin B (CyB) and with or without a recovery period (RP). Therefore the extended exposure has as many as 4 variations outlined as: (1) without CyB – without RP; (2) without CyB – with RP; (3) with CyB – without RP; and (4) with CyB – with RP. In general, an extended exposure experiment is conducted after a negative result is obtained with short-term exposure, so as to maximize the probability of detecting an aneugen or a clastogen. Therefore the sensitivity of this test experiment should be carefully assessed. However, no reliable data are available to choose an appropriate treatment condition for screening MN induction potential using human derived cell lines. The goal of our research was to conduct the MNvit assay with four different extended exposure treatment conditions using TK6 cells to assess the utility of each condition with regard to genotoxicity and cytotoxicity.

Eleven chemicals were chosen for testing in the assay based on their genotoxic modes of action. The first chemical class included DNA reactive clastogens such as alkylating and oxidative agents. These chemicals will induce damage regardless of the cell cycle phase, and the difference in p53 status may cause a different cell cycle regulation between human and rodent derived cell lines: TK6 cells possess a wild-type p53 although hamster derived cells, such as CHL, CHO and V79, are p53 compromised, suggesting that exposure to DNA damaging agents might cause a difference in the cell cycle response and MN induction. The representative chemicals chosen in this class were methylmethanesulfonate, mitomycin C and bleomycin. The second class was the non-DNA reactive antimetabolites and polymerase inhibitors that impact DNA by interacting with the proteins involved in DNA synthesis during DNA replication in the S phase. The representative chemicals chosen in this class were: cytosine arabinofuranoside, aphidicolin and hydroxyurea. The third chemical class included non-DNA reactive aneugens that interact with the mitotic spindle proteins and inhibit spindle formation during metaphase. The chemicals tested in this class were: colcemid, benomyl and thiabendazole. Two negative substances were also included as a reference to assess the validity of the treatment conditions: sodium chloride and nalidixic acid. This chemical set includes the recommended reference chemicals from the OECD Test Guideline 487, MMC, Ara C and two negative substances.

**Table 1**
Summary data of MNvit assay using TK6 cells.

| Chemical | CyB | Recoveryperiod | Range of conc. (ug/mL) | Maximal response | |
|---|---|---|---|---|---|
| | | | | Conc. (ug/mL) | Fold increase |
| Methylmethanesulfonate | | – | 5.39–11.8 | 11.8 | 6.9 |
| | – | + | 5.39–11.8 | 7 | 7.4 |
| | + | – | 5.39–11.8 | N.A. | N.S. |
| | | + | 3.19–7 | 7 | 19.3 |
| Mitomycin C | | – | 0.022–0.06 | 0.06 | 4.6 |
| | – | + | 0.022–0.06 | 0.06 | 18.2 |
| | + | – | 0.022–0.06 | N.A. | N.S. |
| | | + | 0.022–0.06 | 0.06 | 18.1 |
| Bleomycin | | – | 1.17–3.95 | 3.95 | 5.8 |
| | – | + | 0.78–2.63 | 2.63 | 9.2 |
| | + | – | 1.17–3.95 | 2.63 | 1.6 |
| | | + | 1.17–3.95 | 3.95 | 13.9 |
| Cytosine arabinoside | | – | 0.00195–0.00781 | 0.00781 | 2.2 |
| | – | + | 0.000977–0.00781 | 0.00781 | 5.2 |
| | + | – | 0.000977–0.00781 | N.A. | N.S. |
| | | + | 0.000977–0.00781 | 0.00781 | 5.2 |
| Aphidicolin | | – | 0.0119–0.04 | 0.04 | 5.3 |
| | | + | 0.0119–0.04 | 0.04 | 4.3 |
| | + | – | 0.0119–0.04 | N.A. | N.S. |
| | | + | 0.0119–0.04 | 0.04 | 11.7 |
| Hydroxyurea | | – | 3.95–13.3 | 13.3 | 2.1 |
| | – | + | 3.95–13.3 | 13.3 | 6.7 |
| | + | – | 3.95–13.3 | N.A. | N.S. |
| | | + | 3.95–13.3 | 13.3 | 9.9 |
| Colcemid | | – | 0.01–0.016 | 0.016 | 8.7 |
| | – | + | 0.01–0.016 | 0.016 | 14.9 |
| | + | – | 0.01–0.016 | 0.016 | 2.6 |
| | | + | 0.01–0.016 | 0.016 | 12.3 |
| Benomyl | | – | 1.2–2.4 | 2.4 | 10.1 |
| | – | + | 1.2–2.4 | 2.4 | 6.5 |
| | + | – | 1.2–2.4 | 2.4 | 3.9 |
| | | + | 1.2–2.4 | 2.4 | 6.6 |
| Thiabendazole | | – | 20–80 | N.A. | N.S. |
| | – | + | 20–80 | 80 | 1.9 |
| | + | – | 20–80 | N.A. | N.S. |
| | | + | 20–80 | 80 | 1.9 |

–, Without; +, with.
N.A., not applicable.
N.S., not significant increase.