Mutat Res Gen Tox En 831 (2018) 62–68

Contents lists available at ScienceDirect

Mutat Res Gen Tox En

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

Multi-laboratory evaluation of 1,3-propane sultone, *N*-propyl-*N*-nitrosourea, and mitomycin C in the *Pig-a* mutation assay *in vivo*

Young-Shin Chung[a,*], Bumsoo Pak[a], Sehee Han[a], Jiyeon Lee[a], Jiyoung Kim[b], Seng-Min Back[b], Cho-Rong Park[c], Su-Hwan Kim[c], Jong-Kwon Lee[d]

[a] Department of Biotechnology and The Research Institute for Basic Sciences, Hoseo University, Asan, Chungnam, 31499, Republic of Korea
[b] Department of General and Applied Toxicology, Innovative Toxicology Research Center, Korea Institute of Toxicology, KRICT, Daejeon, 34114, Republic of Korea
[c] Biotoxtech Co., Ltd., Cheongju, Chungbuk, 28115, Republic of Korea
[d] Toxicological Screening and Testing Division, National Institute of Food and Drug Safety Evaluation, Cheongju, Chungbuk, 28159, Republic of Korea

ARTICLE INFO

Keywords:
CD59-deficient erythrocytes
CD59-deficient reticulocytes
Flow cytometry
Micronucleus assay
Comet assay

ABSTRACT

The mutagenic potencies of 1,3-propane sultone (PS), *N*-propyl-*N*-nitrosourea (PNU), and mitomycin C (MMC) were investigated in three independent laboratories in Korea using the *Pig-a* assay *in vivo*. Sprague-Dawley rats were treated with vehicle or test substance on three consecutive days. Blood samples were collected for measuring *Pig-a* mutant phenotypes (CD59-deficient erythrocytes, RBC^{CD59-}; CD59-deficient reticulocytes, RET^{CD59-}) on days −1, 15, and 29 after the first treatment. In some studies, blood was collected for determining DNA damage (comet assay) on day 3 and measuring micronucleated reticulocytes (MN-RET) on day 4. Treatment with the alkylating agents PS and PNU induced dose-dependent increases in the frequency of RBC^{CD59-} on days 15 and 29, and caused maximum elevations in the frequency of RET^{CD59-} on day 15. Inter-laboratory comparison of the day 29 *Pig-a* assay data confirmed the mutagenic potencies of PS and PNU, and showed good agreement among the test sites. Treatment with the DNA cross-linker MMC induced increases in the frequencies of RBC^{CD59-} and RET^{CD59-} on days 15 and 29 (all three laboratories). MN-RETs increased significantly in animals treated with PS, PNU, or MMC, but biologically significant increases in DNA damage were observed only with PS and PNU, and not with MMC. The results of this study indicate that the *Pig-a* assay is a sensitive, reproducible method for evaluating the *in vivo* mutagenicity of various test substances, in particular, DNA cross-linkers and alkylating agents. Our limited data on integrating the *Pig-a* assay with the comet and micronucleus assays indicate that a short-term treatment protocol evaluating these three endpoints in a single set of animals may be a robust strategy for evaluating *in vivo* genotoxicity.

1. Introduction

The *Pig-a* assay uses the endogenous *Pig-a* gene as a reporter for evaluating the *in vivo* mutagenicity of chemicals [1]. The *Pig-a* gene codes for one subunit of the *N*-acetylglucosamine transferase complex involved in glycosylphosphatidylinositol (GPI) biosynthesis. Since the glycoside moieties of GPI attach specific marker proteins to the outer surface of hematopoietic cells, inactivating mutations in the *Pig-a* gene result in a deficiency in these cell-surface proteins [2,3]. Wild-type and variant phenotypes of hematopoietic cells are easily detected by treating the cells with fluorescently tagged antibodies against the GPI-anchored proteins, followed by flow cytometric analysis [4,5].

The transgenic rodent assay (TGR) is the only *in vivo* mutation testing method that has an Organisation for Economic Cooperation and Development (OECD) Test Guideline (TG) (OECD TG 488) [6]. The development of the *Pig-a* assay as a regulatory test has attracted global attention and received support from the Health and Environmental Science Institute's Genetic Toxicology Technical Committee (HESI-GTTC) [7], the International Workshop on Genotoxicity Testing (IWGT) [8], and the Japanese Environmental Mutagen Society (JEMS/MMS) [9]. A strength of the *Pig-a* assay is that it can be conducted in any mammalian species, including humans, since the *Pig-a* gene is highly conserved; additional benefits are related to cost, time, and labor efficiency [1]. The small amount (microliters) of blood sample required for endpoint analysis by the assay allows integration with other general toxicology tests (repeat-dose toxicology tests) or with other genotoxicity tests on the same animals [10].

According to the International Conference on Harmonization (ICH) M7 guidelines [11] and the IWGT, the *Pig-a* assay is recommended for follow-up *in vivo* evaluation of chemicals that test positive in the

* Corresponding Author at: Department of Biotechnology, Hoseo University, 20 Hoseo-ro, 79beon-gil, Baebang-eup, Asan, Chungnam, 31499, Republic of Korea.
E-mail address: yschung@hoseo.edu (Y.-S. Chung).

https://doi.org/10.1016/j.mrgentox.2018.05.015
Received 26 February 2018; Received in revised form 13 May 2018; Accepted 13 May 2018
Available online 26 May 2018
1383-5718/

Table 1
Doses, routes of administration and genotoxic profiles of test chemicals.

Test chemicals	CAS No.	Vehicle	Route	Dose Levels mg/kg/day	Genotoxic profiles for Battery tests[a]	Genotoxic Profiles for TGR[b]	Carcinogenicity[c]
1,3-Propane sultone	1120-71-4	water	gavage	20, 40, 80	AMES, CA, in vivo MN; +ve	no data	Group 2A
N-Propyl-*N*-nitrosourea	816-57-9	PBS (pH 6.0)	gavage	30, 60, 120	AMES, CA, in vivo MN; +ve	liver, kidney,bladder & haematopoietic System; +ve	N.C.
Mitomycin C	50-07-7	saline	intra-peritoneal	0.5, 1, 2	AMES, CA, in vivo MN; +ve	liver & haematopoietic system; +ve	Group 2B

+ve, Positive results for genotoxicity; TGR, transgenic rodent assay; AMES, bacterial reverse mutation assay; CA, *in vitro* chromosomal aberration assay, MN, micronucleus test; N.C., not classified.
[a] From reference [31].
[b] From reference [33].
[c] IARC Classification [27,43].

bacterial reverse mutation test (OECD TG 471) [12] or the mouse lymphoma cell assay (MLA, OECD TG 490) [13]. Since genotoxicity tests generally detect only part of the spectrum of possible genotoxic responses, analysis of the outcomes of a battery of tests is recommended for evaluating the complete genotoxic potential of a test article [14]. Integration of the *in vivo Pig-a* assay with the reticulocyte micronucleus or comet assay as part of short- and long-treatment protocols may provide a good model for a battery of *in vivo* tests.

Among the chemicals that have been tested in the *Pig-a* assay are alkylating agents, aromatic nitro/amino compounds, nanomaterials, and polycyclic aromatic hydrocarbons. Based on these results, the *Pig-a* assay appears to be a reproducible and sensitive test for evaluating *in vivo* mutagenicity [8]. A limitation of the assay is that adequate exposure of bone marrow is required for detecting the variant phenotypes of hematopoietic cells [1,8]. Another limitation may be the detection of clastogens/anuegens [8]. Some clastogens, such as cisplatin [15,16] and cyclophosphamide [17], were positive in the *Pig-a* assay, but others, such as azidothymidine [18], 1,2-dimethylhydrazine [19], 5-fluorouracil [20], hydroxyurea [17], and vinblastine sulfate [21] were negative. Studies of chemicals with diverse modes of action will be helpful in evaluating the *Pig-a* assay as a regulatory test.

This report presents the results of a collaborative study conducted by the Ministry of Food and Drug Administration (MFDA), Korea, for evaluating the efficiency of the *Pig-a* assay as a regulatory test. Two GLP laboratories specializing in genotoxicity testing and one university laboratory having animal facilities in compliance with GLP participated in a study of the performance of the *Pig-a* assay. Two alkylating agents (1,3-propane sultone (PS) and *N*-propyl-*N*-nitrosourea (PNU)) and one DNA cross-linker (mitomycin C (MMC)) were selected to assess the efficiency of the *Pig-a* assay in evaluating mutagenicity. A short-term treatment protocol was chosen, and, in some studies, the *Pig-a* test was integrated with the micronucleus test and comet assay, using the same animals.

2. Materials and methods

2.1. Chemicals

The three test articles and most reagents were purchased by one participating laboratory and distributed to the other two participating laboratories: 1,3-propane sultone (CAS 1120-71-4; Sigma-Aldrich, St. Louis, MO, USA), *N*-propyl-*N*-nitrosourea (CAS 816-57-9; Toronto Research Chemicals Inc., Toronto, ON, Canada), mitomycin C (CAS 50-07-7; TOCRIS, Avonmouth, Bristol, UK), PBS, pH 7.2 (Life Technologies, New York, USA), anticoagulant solution, anti-CD59-PE, anti-CD61-PE, and SYTO®13 (Litron Laboratories, Rochester, NY, USA), Lympholyte®-Mammal (CedarLane, Burlington, NC, USA), anti-PE microBeads, LS columns and QuadroMACS™ Separator (Mitenyi Biotec, Bergisch Gladbach, Germany), CountBright™ Absolute Count Beads (Invitrogen, Carlsbad, CA, USA). FBS, saline, and deionized water were independently purchased by participating laboratories. SYBR Gold, comet slides and low-melting agarose were purchased from TREVIGEN Co. (Gaithersburg, MD, USA), and acridine orange, dimethyl sulfoxide, Triton X-100 were purchased from Sigma-Aldrich.

2.2. Animals, treatments, and blood collection

All animal studies were approved independently by the Animal Care and Use Committees of the participating institutes and were conducted in accordance with the most current version of the Korean Association of Laboratory Animal Science guidelines. Crl:CD (SD) male rats (CD®IGS, 6-week-old, SPF) were purchased by each participant from the same distributor, Orientbio (Seongnam, Gyenggi-do, Korea). Rats were acclimated for approximately 7–10 d prior to the treatments. Two or three rats were housed per cage, and food and water were provided *ad libitum*. Ambient temperature 22 ± 3 °C, relative humidity 50 ± 10%, and photoperiod 12 h were maintained throughout the study.

Rats were exposed to the test chemicals once per day for three consecutive days, at 24-h intervals. Genotoxicity profiles, administered doses, routes of administration, and vehicles for each chemical are presented in Table 1. Maximum doses of PS and MMC were selected from previous reports [22,31] and a dose-finding test was performed to establish the highest dose of PNU. Blood samples were collected on days −1, 15, and 29 after the first treatment for quantifying *Pig-a* gene mutant frequency in hematopoietic cells, within 3–4 h after the third administration (on day 3) for evaluating DNA damage, and 24 h after the third administration (on day 4) for assessing micronucleated reticulocytes. After warming the rats in a cabinet or with an infrared light, the rat tail vein was pierced with a 25-gauge needle coated with anticoagulant and free-flowing blood was collected into K_2EDTA-coated microtubes.

2.3. Pig-a assay

The *Pig-a* assay was conducted within 24 h of blood collection as indicated in the MutaFlow kit manual (Rat, tube-based version 140403) and previous reports [17,23], with minor modification. In brief, leukocytes and platelets in blood samples were depleted using Lympholyte®-Mammal. Wild-type and mutant phenotype erythrocytes were discriminated by labelling with PE-conjugated anti-CD59, and reticulocytes were stained with SYTO®13. Magnetic separation was performed for enrichment of erythrocytes with mutant phenotypes. For flow cytometric analysis, an instrument calibration standard containing a high proportion of mutant-mimic cells was generated on each day of data acquisition and used for defining the location of CD59-negative erythrocytes. The following instruments were used for *Pig-a* data acquisition; BD AccuriC6™ running AccuriC6 pro in Hoseo University (HOSEO), BD FACSCalibur™ running CellQuest Pro in the Korean