



# Evaluation of the PIGRET assay as a short-term test using a single dose of diethylnitrosamine



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## ARTICLE INFO

### Article history:

Received 1 February 2016

Accepted 15 February 2016

Available online 6 March 2016

### Keywords:

Collaborative study in Japan

RBC *Pig-a* assay

PIGRET assay

Diethylnitrosamine

## ABSTRACT

The PIGRET assay, which was developed as the *Pig-a* assay in reticulocytes, can detect mutagenicity of compounds earlier than the *Pig-a* assay in total red blood cells (RBC; RBC *Pig-a* assay). The usefulness of the PIGRET assay as a short-term test has been confirmed in a collaborative study in Japan with 24 chemicals. One of these chemicals, diethylnitrosamine (DEN), which mainly induces liver tumors in both sexes of rats, was tested. To determine the appropriate doses, DEN was dissolved in physiological saline and administered orally with a single dose to male 8-week-old Sprague-Dawley rats in a preliminary dose-range finding study. As a result, all of the animals died at doses of 300 and 600 mg/kg. Therefore, 37.5, 75, and 150 mg/kg doses were set for the main study (the RBC *Pig-a* and PIGRET assays). The results showed no statistically significant increase in the mutant frequency (MF) of CD59-negative cells in the groups treated with DEN for the entire test period; however, the positive control *N*-ethyl-*N*-nitrosourea (ENU) produced positive results. Some hematogenic effects were indicated by the significant increase of the percentage of reticulocytes in the medium and at high doses on Day 28. The decrease in the body weight on Days 2–4 in the main study and the mortality in the preliminary dose range-finding study indicated that appropriate doses were used in the main study. Although DEN is a known genotoxic carcinogen in the liver, our negative results in the RBC *Pig-a* and PIGRET assays indicated that there is no substantial mutagenicity in hematopoietic cells under the conditions using a single dose. The PIGRET assay detected the mutagenicity of ENU one week earlier than the RBC *Pig-a* assay, indicating the usefulness of the PIGRET assay as a short-term test.

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## 1. Introduction

The *Pig-a* (phosphatidylinositol glycan complementation group A) assay can detect glycosylphosphatidylinositol-anchored protein-deficient cells resulting from a *Pig-a* gene mutation [1,2]. This assay has been mainly applied to red blood cells (RBCs) in rodents by flow cytometry to detect *in vivo* genotoxicity as induction of CD59-negative cells. The usefulness of this assay was evaluated in a collaborative study in the USA [3]. Furthermore, the *Pig-a* assay using reticulocytes (RETs), the PIGRET assay, was developed by Kimoto et al. [4,5] using RETs obtained by the magnetic enrichment of CD71-positive RETs from peripheral blood to evaluate the early stage of mutation after dosing. In the previous

collaborative study for the *Pig-a* assay in Japan [6–12], authors [6,7] suggested that the PIGRET assay has the potential to detect mutagenic compounds earlier than the RBC *Pig-a* assay.

The *Pig-a* assay is now expected to be adopted as a new OECD test guideline. The PIGRET assay also has the potential to be included in the guideline for its usefulness as a short-term test using a single dose. Therefore, a collaborative study was organized by the Mammalian Mutagenicity Study Group, a subgroup of the Japanese Environmental Mutagen Society, to evaluate the advantage of the PIGRET assay over the RBC *Pig-a* assay using 24 chemicals. One of these chemicals, diethylnitrosamine (DEN), which mainly induces liver tumors in multiple species, including rodents [13], was tested in our laboratory.

## 2. Materials and methods

The standard protocol was distributed by Dr. K. Horibata (National Institute of Health Sciences), who is one of the representatives of the collaborative study. The detailed materials and methods are described here.

Abbreviations: *Pig-a*, phosphatidylinositol glycan complementation group A; DEN, diethylnitrosamine; ENU, *N*-ethyl-*N*-nitrosourea; MF, mutant frequency; RBC, red blood cell; RET, reticulocyte.

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## 2.1. Chemicals

DEN (CAS No. 55-18-5) was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), and *N*-ethyl-*N*-nitrosourea (ENU, CAS No. 759-73-9) was obtained from Sigma-Aldrich (MO, USA). K2-ethylenediaminetetraacetic acid (K2-EDTA) was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free Dulbecco's phosphate-buffered saline (DPBS, pH 7.4) was from GIBCO (Life Technologies, Inc., NY, USA). Physiological saline was from Otsuka Pharmaceutical Factory, Inc., (Tokushima, Japan). IMag<sup>TM</sup> Buffer, IMag<sup>TM</sup> PE Particles Plus-DM, anti-rat CD59 antibody (clone TH9, FITC-conjugated), anti-rat CD71 antibody (clone OX-26, PE-conjugated), and anti-rat erythroid marker antibody (clone HIS49, APC-conjugated) were from BD Biosciences (Tokyo, Japan).

## 2.2. Animals

The study was conducted in accordance with the Animal Care and Use Program at the Institute of Environmental Toxicology. Male animals only were used in this study as discussed in the summary paper of this collaborative study [14]. Male Sprague-Dawley [CrI: CD (SD)] rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan); randomly assigned into groups of three animals each; acclimated for six days in an animal room (temperature, 22 ± 3 °C; humidity, 50 ± 20%; 12-h light/dark cycle); and underwent drug administration at eight weeks of age. Standard laboratory pellet chow MF (Oriental Yeast Co., Ltd., Tokyo, Japan) in stainless steel feeding baskets and tap water through an auto-watering system were available *ad libitum*.

## 2.3. Animal treatment

All of the animals were orally administered with a single dose at a volume of 10 mL/kg body weight by gavage. A preliminary dose range-finding study was performed using four doses of 75, 150, 300, and 600 mg/kg using three animals per group based on the known LD<sub>50</sub> for DEN (280 mg/kg) [15]. As a result, all of the animals died at doses of 300 and 600 mg/kg within one week. Therefore, the highest dose of 150 mg/kg was used for the main study. Physiological saline was used as the DEN solvent and was administered to the rats (n = 6) as the vehicle control. ENU was dissolved in DPBS (pH adjusted to 6.0–6.1 by HCl) at a concentration of 4 mg/mL and administered to the rats (n = 3) as the positive control. Blood sampling (approximately 200 µL) was performed on days –3, 7, 14, and 28 (day = 0: the day of administration) via the jugular vein under isoflurane inhalation anesthesia. The obtained blood was placed into a tube with 20 µL of an anticoagulant (12 mg/mL K2-EDTA solution), mixed immediately, and stored under cold conditions until use within one week. Clinical signs and body weight changes were recorded every day for the first week after the administration and once per week thereafter.

## 2.4. RBC pig-a assay

Three microliters of the blood suspended in 200 µL of DPBS were mixed with a working antibody solution (1 µg of an anti-rat CD59 antibody and 0.13 µg of an anti-rat erythroid antibody solution). The mixture was incubated for 1 h in the dark at room temperature, and then the mixture was vortexed and centrifuged at 1680g for 5 min at room temperature. After the supernatant was aspirated, the pellet was tapped repeatedly and re-suspended in 500 µL of DPBS. The obtained samples were stored in the cold and analyzed using a FACSVerse flow cytometer (BD Biosciences) for the detection of CD59-negative cells, the gating region of which had been set not according to the standard protocol to include 98.9–99.1%

but according to a personal communication from Dr. K. Horibata to include 98.5–99.1% of cells labeled with anti-rat erythroid marker antibody due to a specification of the software used. This extension of the range had no effect on data acceptability.

## 2.5. PIGRET assay

To obtain reticulocyte-enriched samples, 80–150 µL of blood/EDTA was mixed with 195 µL of DPBS and 1 µg of an anti-rat CD71 antibody for 15 min at 4 °C in a refrigerator. The labeled cells were washed with 2 mL of IMag<sup>TM</sup> Buffer and centrifuged (1680g, 5 min). After the supernatant was aspirated, the obtained cells were mixed with 50 µL of BD IMag<sup>TM</sup> PE Particles Plus-DM and incubated for 15 min at 4 °C in a refrigerator, and then the fraction of CD71-positive cells was enriched using a BD IMagnet<sup>TM</sup> magnetic stand (BD Biosciences) according to the manufacturer's suggested positive selection method. The enriched samples were labeled with an anti-rat erythroid marker antibody and anti-CD59 antibodies as indicated in the methods for the RBC *Pig-a* assay, with the exceptions that the incubation time and temperature for labeling enriched RETs were 30 min incubation under cold conditions, respectively.

The percentage of RET was measured to assess the toxicity to erythropoiesis. Three microliters of the blood suspended in 200 µL of DPBS was mixed with a working antibody solution (1 µg of an anti-rat CD71 antibody and 0.13 µg of an anti-rat erythroid antibody). The mixture was incubated for 30 min at room temperature, vortexed, and centrifuged at approximately 1680g for 5 min at room temperature. After the supernatant was aspirated, the pellet was tapped repeatedly and re-suspended in 500 µL of DPBS. The obtained samples were analyzed by flow cytometry.

## 2.6. Statistics

RBC *Pig-a* mutant frequencies (RBC *Pig-a* MFs) are expressed as the number of CD59-negative cells per 1 million RBCs labeled with the anti-rat erythroid marker antibody. RET *Pig-a* mutant frequencies (RET *Pig-a* MFs) are expressed as the number of CD59-negative cells per 1 million RETs labeled both with CD71 and the anti-rat erythroid marker. The % RET was expressed as the ratio of the RETs per ten thousand RBCs labeled with the anti-rat erythroid marker.

Statistical analyses of the *Pig-a* MF data in the DEN-treated group were performed at Teijin Pharma Limited using EXSUS Ver. 7.7.1 (CAC EXICARE Corporation, Tokyo, Japan) as follows. An offset of 0.1 was added to each *Pig-a* MF value because *Pig-a* MFs of zero were occasionally observed and then log (10) transformed. Transformed *Pig-a* MF values were analyzed by Bartlett's test for homogeneity of variance among the groups. If the group variance was determined to be homogeneous, Dunnett's multiple comparison test was employed. If Bartlett's test indicated heterogeneous variance, the nonparametric Dunnett's multiple comparison test (Steel test) was used. Significance was evaluated at the 5% level using a one-tailed test for increases relative to the vehicle control.

Statistical analyses of the *Pig-a* MF data in the ENU-treated groups, the body weight, and % RET in all groups were performed at our laboratory using the IBM SPSS Statistic software package as described in the methods above with the exception of using a two-tailed test at the 1% level instead of a one-tailed test at the 5% level.

## 3. Results

### 3.1. Body weight gain or loss and clinical signs of toxicity

Body weight gain or loss is shown in Fig. 1 (A) and (B). Animals treated with DEN at the dose of 150 mg/kg showed significantly

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