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Evaluation of mutagenicity of acrylamide using RBC *Pig-a* and PIGRET assays by single peroral dose in rats

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ABSTRACT

The *Pig-a* gene mutation assay, a powerful tool for evaluating *in vivo* genotoxicity, is based on flow cytometric enumeration of red blood cells (RBCs), which are deficient in glycosylphosphatidylinositol anchored proteins caused by mutation(s) in the *Pig-a* gene. Various approaches for measuring cells with mutated *Pig-a* gene have been developed. The *Pig-a* assay targeting concentrated reticulocytes – the PIGRET assay – has the potential to detect genotoxicity in early stages of the study. To verify the potential and usefulness of the PIGRET assay for short-term testing, we conducted a joint research with the Mammalian Mutagenicity Study (MMS) Group of the Japanese Environmental Mutagen Society. As part of this study, we evaluated the genotoxicity of a single oral administration of acrylamide (AA) at 25, 50, 100, 137.5, and 175 mg/kg using the PIGRET and *Pig-a* assays targeting RBCs (RBC *Pig-a* assay) at 7, 14, and 28 days after dosing. Toxic effects induced by AA, such as hind limb weak-paralysis, reduction of body weight gain, and reticulocytosis, were observed in AA-treated groups. However, we detected no significant increases in *Pig-a* mutant frequencies using either the PIGRET or RBC *Pig-a* assay. Therefore, we concluded that the genotoxicity of AA could not be detected by these assays under our experimental conditions.

1. Introduction

Because gene mutations are implicated in the etiology of cancer and other diseases, *in vivo* genotoxicity tests are important as public health management tools. The recently developed *Pig-a* gene mutation assay is a powerful and useful tool for evaluating *in vivo* genotoxicity. Because the *Pig-a* gene is on the X-chromosome and involves the first step of glycosylphosphatidylinositol (GPI) anchor biosynthesis, mutation(s) in the *Pig-a* gene can result in the loss of expression of GPI-anchored proteins on the cell membrane; this phenotype can be detected by flow cytometry [1–3]. Various approaches for measuring *Pig-a* mutant cells by flow cytometry have been developed, particularly those focused on peripheral red blood cells (RBCs) and reticulocytes (RETs) [4–14]. Although the fluorescent SYTO 13 dye was used to label RETs in many reports, there was a limited capacity for counting RETs by flow cytometry. Conversely, the PIGRET assay, a recently developed assay for measuring *Pig-a* mutant RETs, is capable of allowing flow cytometric cell counting of $>1 \times 10^6$ RETs for the *Pig-a* mutant by concentrating RETs [15,16]. Additionally, due to the characteristics of erythropoiesis, the PIGRET assay can detect increases in *Pig-a* mutant frequency (MF) sooner after exposure compared with the *Pig-a* assay targeting RBCs (RBC *Pig-a* assay) [15–21]. To evaluate the usefulness of the PIGRET assay as a short-term genotoxicity test, we evaluated the genotoxicity of acrylamide (AA) as part of the joint study organized by the Mammalian Mutagenicity Study Group (MMS), a subgroup of the Japanese Environmental Mutagen Society (JEMS).

The International Agency for Research on Cancer classifies AA as 2A, a probable human carcinogen [22]. The *in vivo* genotoxicity of AA has been demonstrated by various rodent genotoxicity tests, including micronucleus (MN) tests in peripheral blood, gene mutation, and comet assays in various organs [23–30]. AA is metabolized to glycidamide (GA), presumably by cytochrome P450 2E1, which quickly reacts with cellular DNA and proteins [31–33] and forms several specific DNA adducts [34–36]. Therefore, GA is considered responsible for the majority of AA genotoxicity [28,37,38]. However, it was mentioned that *in vivo* AA genotoxicity analyzed by

Abbreviation: %RET, percent of reticulocyte; AA, acrylamide; APC, allophycocyanin; ENU, *N*-ethyl-*N*-nitrosourea; FITC, fluorescein isothiocyanate; GA, glycidamide; GPI, glycosylphosphatidylinositol; HSC, hematopoietic stem cell; JEMS, the Japanese Environmental Mutagen Society; LD50, median lethal dose; MN, micronucleus; MF, mutant frequency; MMS, Mammalian Mutagenicity Study Group; PBS, phosphate-buffered saline; PE, phycoerythrin; PNH, paroxysmal nocturnal hemoglobinuria; RBC, red blood cell; RET, reticulocyte.

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Pig-a assays was negative or inconclusive over 28 consecutive days of dosing [39,40]. Additionally, AA genotoxicity induced by acute dosing has never been analyzed by *Pig-a* assays.

In this report, to understand whether single-dosed AA genotoxicity can be detected by the *Pig-a* assays, we administered a single dose of AA which was increased up to the median lethal dose (LD50) and analyzed the genotoxicity by the RBC *Pig-a* and PIGRET assays using blood samples collected from male rats at pre-dosing (baseline), and 7, 14, and 28 days after dosing.

2. Materials and methods

2.1. Animals

F344/NSlc male rats were obtained from Japan SLC (Shizuoka, Japan). Animals were housed individually under specific pathogen-free conditions with a 12-h light–dark cycle. Food (CRF-1 pellet feed, Oriental Yeast Co., Ltd., Tokyo, Japan) and water were available *ad libitum*. Animal experiments were conducted in accordance with regulations of the Animal Care and Use Committee of the National Institute of Health Sciences, Tokyo, Japan. Male animals only were used in this study as discussed in the summary paper of this collaborative study [41].

2.2. Chemicals

N-ethyl-*N*-nitrosourea (ENU, CAS No.: 759-73-9) and AA (CAS No.: 79-06-1) were purchased from Sigma–Aldrich Japan (Tokyo, Japan) and dissolved in phosphate-buffered saline (PBS; pH 6.0) and distilled water (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan), respectively. We obtained an anti-rat CD59 [clone TH9, fluorescein isothiocyanate (FITC)-conjugated], anti-rat CD71 [clone OX-26, phycoerythrin (PE)-conjugated], and anti-rat erythroid marker [clone HIS49, allophycocyanin (APC)-conjugated] antibodies from BD Biosciences (Tokyo, Japan).

2.3. Dose levels and treatment

At eight weeks of age, six male rats per group were given a single oral administration of AA (25, 50, 100, 137.5, or 175 mg/kg) or distilled water (the negative control). Because it has been reported that the LD50 of AA was 175 mg/kg seven days after a single oral dosing using F344 male rats [42], we set the highest dose at 175 mg/kg AA in our experimental conditions. ENU (40 mg/kg) was administered to three male rats per group for the positive control. The administration day was considered Day 0. During the experimental period, the animals were weighed on Days-2, 0, 7, 14, and 28. The general condition of the animals was examined twice daily on Day 0 and once daily on Days 7, 14, and 28. On Days-2, 7, 14, and 28, 120 μL of peripheral blood was withdrawn from a tail vein, immediately transferred into EDTA (dipotassium salt)-coated Microtainer Tubes (BD Biosciences), and used for the RBC *Pig-a* and PIGRET assays.

2.4. Analysis of percent of reticulocytes (%RET)

Three microliters of blood were suspended in 0.2 mL PBS and labeled with anti-rat CD71 (1 μg) and anti-rat erythroid marker (0.133 μg) antibodies. The cells were incubated for 30 min in the dark at room temperature, centrifuged (1680 × *g*, 5 min), resuspended in 1 mL PBS, and examined using a FACS Canto II flow cytometer (BD Biosciences).

2.5. Pig-a *mutation assays*

The RBC *Pig-a* and PIGRET assays were performed as previously described [2,15–21]. For the RBC *Pig-a* assay, 3 μL of blood was labeled with anti-rat CD59 (1 μg) and anti-rat erythroid marker (0.133 μg) antibodies. Approximately 1×10^6 erythroid marker-positive cells were analyzed using a FACS Canto II flow cytometer (BD Biosciences) for the presence of surface CD59, and the *Pig-a* MFs of RBCs (RBC *Pig-a* MFs) were calculated as previously described [16,21]. For the PIGRET assay, 80 μL of blood was labeled with 1 μg of PE-conjugated anti-rat CD71 antibody, and the samples were enriched for CD71-positive cells by processing with a BD IMagnet magnetic stand (BD Biosciences) according to the manufacturer's instructions. The enriched samples were labeled with HIS49 and anti-CD59 antibodies, and *Pig-a* MFs of CD71-positive RETs (RET *Pig-a* MFs) were examined using a FACS Canto II flow cytometer (BD Biosciences) as previously described [16,21]. On the gating strategies of the flow cytometer, to avoid artifactually inflating both RBC and RET *Pig-a* MFs, we refined the gate for *Pig-a* mutant RBCs or *Pig-a* mutant RETs as the area encompassing a maximum of 99.0% of the lower RBC or RET FITC staining intensities only, as previously described [8,16].

2.6. Calculations and statistical analyses

RBC *Pig-a* MFs are expressed as the number of CD59-negative cells per one million HIS49-positive RBCs. RET *Pig-a* MFs are expressed as the number of CD59-negative cells per one million CD71 and HIS49 double-positive RETs.

Statistical analyses of *Pig-a* MF data 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's 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 body weight gain and %RET data were performed using Excel Statistics 2012 (Social Survey Research Information, Tokyo, Japan) as follows. Distributions were tested by Bartlett's test. If the distributions were normal, one-way ANOVA followed by Dunnett's (pair-wise comparisons of the frequencies in treated groups to the vehicle control group, two-sided) *post-hoc* test were applied. Otherwise, Kruskal–Wallis test followed by Steel's (pair-wise comparisons of the frequencies in treated groups to the vehicle control group, two-sided) *post-hoc* test were applied for analysis.

The data obtained from ENU-treated rats were excluded from the statistical analyses.

3. Results

3.1. Body weight and general condition

The animals in all treatment groups survived. Significant difference in body weight gain was detected in the 175-mg/kg AA-treated group on Day 7 (Fig. 1). Hindlimb weak-paralysis was observed in the 100-, 137.5-, and 175-mg/kg AA-treated groups on Day 7, and this paralysis disappeared on Day 14 (data not shown).

3.2. Frequency of RETs

Significant increases in reticulocytosis were observed in the 100-, 137.5-, and 175-mg/kg AA-treated groups on Day 14 (Fig. 2).

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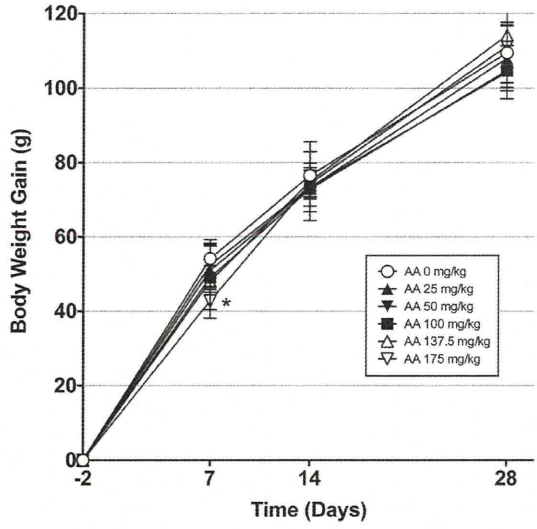

Fig. 1. Body weight gain. Body weight gains were calculated by subtracting the body weight values on Day-2 from the body weight values at each time point. The mean ± SD at each time point is shown. The data obtained from ENU-treated rats were excluded from the statistical analysis. * indicate $p < 0.05$ by Dunnett's test. Statistical significance was only observed in the highest-dose of AA-treated group on Day 7. The data of body weight gain on Day 0 was omitted because of no association with the data of %RET, RBC *Pig-a* MF and RET *Pig-a* MF.

Reticulocytosis was sustained in the 137.5- and 175-mg/kg AA-treated groups on Day 28 (Fig. 2).

3.3. *RBC* Pig-a *and PIGRET assays*

RBC *Pig-a* MFs in the ENU-treated rats were clearly increased on Day 14 and Day 28, and the increase was time-dependent (Table 1). Conversely, we detected no significant differences in RBC *Pig-a* MFs in any of the AA-treated groups (Table 1 and Fig. 3). In the case of RET *Pig-a* MFs, clear increases of RET *Pig-a* MF were detected on Day 7 and later in the ENU-treated group (Table 1). The mean values of RET *Pig-a* MFs in the 137.5- and 175-mg/kg AA-treated groups

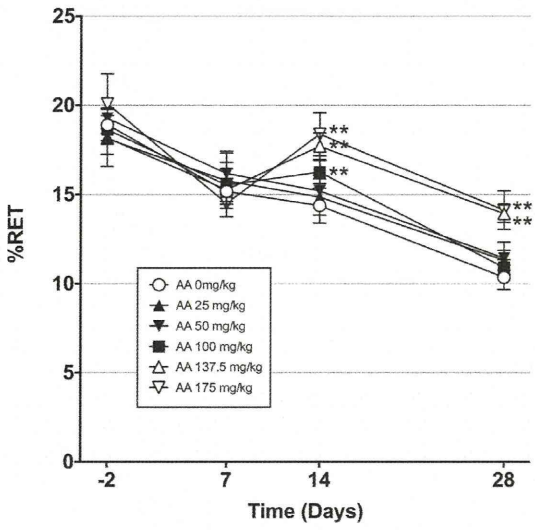

Fig. 2. %RET. The mean ± SD at each time point is shown. The data obtained from ENU-treated rats were excluded from the statistical analysis. ** indicate $p < 0.01$ by Dunnett's test. Statistical significances were observed in the 100-, 137.5-, and 175-mg/kg AA-treated groups on Day 14 and in the 137.5- and 175-mg/kg AA-treated groups on Day 28.

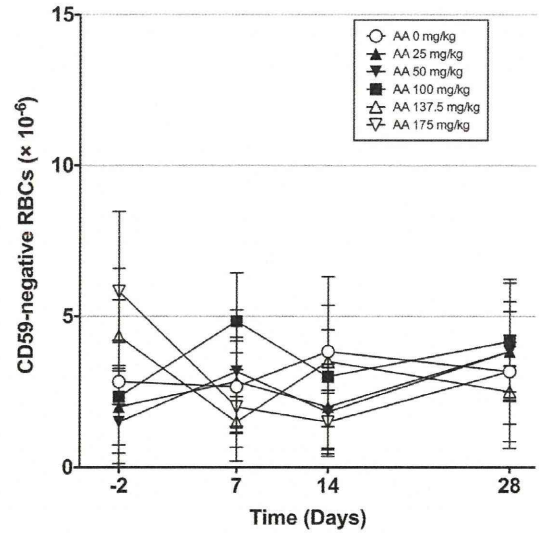

Fig. 3. *Pig-a* mutant frequencies of RBCs induced by acrylamide. >1 × 10^6 RBCs were analyzed by flow cytometry for the presence of CD59 on the surface of RBCs, and the frequencies of CD59-negative RBCs (×10^{-6}) were calculated. The mean ± SD at each time point is shown. No statistical significance was observed among the AA-treated groups.

were marginally increased at 28 days after the treatment, but we detected no significant differences in RET *Pig-a* MFs in any of the AA-treated groups (Table 1 and Fig. 4).

4. Discussion

In this study, we analyzed the genotoxicity of single-dosed AA by the RBC *Pig-a* and PIGRET assays as part of the collaborative study organized by MMS, a subgroup of JEMS. It is known that AA is a neurotoxin; therefore we investigated motor disturbances and observed weak-paralysis in the hind limb in the 100-, 137.5-, and 175-mg/kg AA-treated groups on Day 7. Significant reduction in body weight gain was detected in the 175-mg/kg AA-treated groups

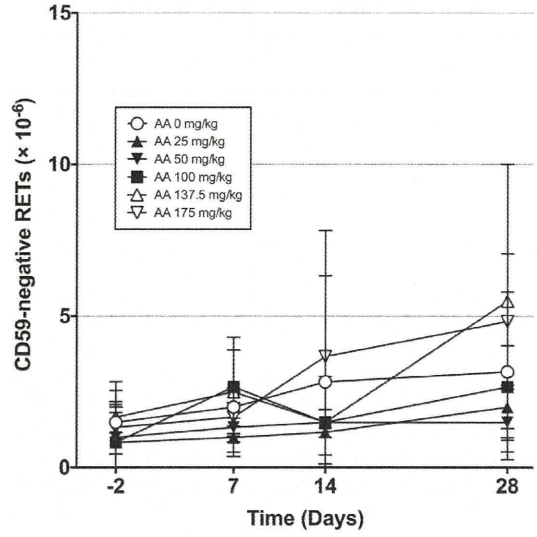

Fig. 4. *Pig-a* mutant frequencies of RETs induced by acrylamide. Peripheral blood was withdrawn from the tail vein and RETs were concentrated. >1 × 10^6 RETs were analyzed by flow cytometry for the presence of CD59 on the surface of RETs, and the frequencies of CD59-negative RETs (×10^{-6}) were calculated. The mean ± SD at each time point is shown. No statistical significance was observed among the AA-treated groups.

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Table 1
Frequencies of CD59-negative cells determined by the RBC *Pig-a* and PIGRET assays following single AA dosing.

Pig-a mutant frequency ($\times 10^{-6}$)									
		RBC *Pig-a* assay				PIGRET assay			
Group	Animal No.	Day-2	Day 7	Day 14	Day 28	Day-2	Day 7	Day 14	Day 28
Control	1	1.00	4.00	6.00	3.00	1.00	2.00	1.00	1.00
	2	1.00	4.00	3.00	3.00	3.00	2.00	1.00	1.00
	3	3.00	3.00	6.00	3.00	2.00	3.00	1.00	4.00
	4	3.00	2.00	1.00	5.00	1.00	2.00	1.00	2.00
	5	8.00	0.00	1.00	2.00	2.00	1.00	0.00	3.00
	6	1.00	3.00	6.00	3.00	0.00	2.00	13.00	8.00
	Ave.	2.83	2.67	3.83	3.17	1.50	2.00	2.83	3.17
	S.D.	2.71	1.51	2.48	0.98	1.05	0.63	5.00	2.64
AA	1	2.00	2.00	4.00	4.00	0.00	1.00	2.00	4.00
25 mg/kg	2	2.00	3.00	3.00	8.00	0.00	1.00	1.00	1.00
	3	0.00	1.00	1.00	2.00	0.00	2.00	0.00	1.00
	4	4.00	5.00	0.00	1.00	2.00	0.00	1.00	2.00
	5	2.00	2.00	2.00	4.00	2.00	1.00	1.00	2.00
	6	2.00	4.00	2.00	4.00	1.00	1.00	2.00	2.00
	Ave.	2.00	2.83	2.00	3.83	0.83	1.00	1.17	2.00
	S.D.	1.26	1.47	1.41	2.40	0.98	0.63	0.75	1.10
AA	1	1.00	5.00	0.00	4.00	1.00	2.00	2.00	0.00
50 mg/kg	2	1.00	1.00	3.00	6.00	1.00	2.00	0.00	1.00
	3	2.00	3.00	1.00	2.00	1.00	1.00	1.00	1.00
	4	5.00	3.00	2.00	4.00	3.00	0.00	2.00	3.00
	5	0.00	1.00	4.00	3.00	0.00	2.00	4.00	1.00
	6	0.00	6.00	1.00	4.00	0.00	1.00	0.00	3.00
	Ave.	1.50	3.17	1.83	3.83	1.00	1.33	1.50	1.50
	S.D.	1.87	2.04	1.47	1.33	1.10	0.82	1.52	1.22
AA	1	2.00	3.00	6.00	4.00	3.00	3.00	3.00	3.00
100 mg/kg	2	0.00	5.00	1.00	5.00	0.00	1.00	0.00	1.00
	3	1.00	7.00	6.00	1.00	1.00	2.00	3.00	5.00
	4	2.00	6.00	2.00	4.00	0.00	4.00	1.00	2.00
	5	5.00	5.00	2.00	4.00	1.00	1.00	2.00	2.00
	6	4.00	3.00	1.00	7.00	0.00	5.00	0.00	3.00
	Ave.	2.33	4.83	3.00	4.17	0.83	2.67	1.50	2.67
	S.D.	1.86	1.60	2.37	1.94	1.17	1.63	1.38	1.37
AA	1	4.00	1.00	4.00	1.00	2.00	4.00	1.00	3.00
137.5 mg/kg	2	8.00	2.00	3.00	3.00	2.00	1.00	3.00	2.00
	3	1.00	3.00	3.00	2.00	2.00	2.00	0.00	6.00
	4	4.00	1.00	5.00	6.00	2.00	4.00	0.00	13.00
	5	5.00	1.00	4.00	2.00	1.00	1.00	2.00	1.00
	6	4.00	1.00	2.00	1.00	1.00	3.00	3.00	8.00
	Ave.	4.33	1.50	3.50	2.50	1.67	2.50	1.50	5.50
	S.D.	2.25	0.84	1.05	1.87	0.52	1.38	1.38	4.51
AA	1	8.00	5.00	2.00	3.00	2.00	1.00	1.00	3.00
175 mg/kg	2	7.00	0.00	0.00	7.00	0.00	3.00	5.00	3.00
	3	7.00	1.00	1.00	2.00	4.00	2.00	7.00	3.00
	4	8.00	2.00	2.00	4.00	1.00	2.00	4.00	5.00
	5	2.00	1.00	3.00	0.00	1.00	1.00	0.00	7.00
	6	3.00	3.00	1.00	3.00	0.00	1.00	5.00	8.00
	Ave.	5.83	2.00	1.50	3.17	1.33	1.67	3.67	4.83
	S.D.	2.64	1.79	1.05	2.32	1.51	0.82	2.66	2.23
ENU	1	5.00	11.00	71.00	116.00	3.00	40.00	101.00	183.00
40 mg/kg	2	14.00	18.00	69.00	100.00	3.00	48.00	129.00	177.00
	3	8.00	9.00	65.00	104.00	4.00	47.00	120.00	157.00
	Ave.	9.00	12.67	68.33	106.67	3.33	45.00	116.67	172.33
	S.D.	4.58	4.73	3.06	8.33	0.58	4.36	14.29	13.61

on Day 7 (Fig. 1). Additionally, reticulocytosis was observed in the 100-, 137.5-, and 175-mg/kg AA-treated groups on Day 14 (Fig. 2). These results indicated that rats were sufficiently exposed to AA in this experiment; however, the results of administration of AA to F344 male rats were negative or inconclusive after both RBC *Pig-a* and PIGRET assays were performed under our experimental conditions (Table 1 and Figs. 3 and 4).

Pig-a assays, including the RBC *Pig-a* and PIGRET assays, are based on the detection of GPI-anchored proteins on the cell surface [2,3,15,43]. The *Pig-a* gene is involved in the synthesis of GPI anchors that link various protein markers to the cell surface. It has been shown that paroxysmal nocturnal hemoglobinuria (PNH) is caused by somatic *PIG-A* mutations in hematopoietic stem cells (HSCs) and aerolysin-resistant HSCs from a patient with PNH exhibited clonal *PIG-A* mutations [44,45]. Additionally, it has been considered that the absence of GPI-anchored protein in RBCs is caused by mutations in the *Pig-a* gene of nucleated erythroid precursors and/or of HSCs [3]. These findings indicated that expression of GPI-anchored CD59 appears to be dependent on the *Pig-a* gene mutations in erythroid precursors and/or of HSCs in bone marrow. Accordingly, we considered that our results reflected genotoxicity of AA in bone marrow.

The *in vivo* genotoxicity of AA has been demonstrated. A recent study using young *versus* adult *gpt* delta F344 male rats showed

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that MN frequencies in bone marrow and *gpt* MFs in testes were significantly increased only in 3-week-old young rats with 28 days of consecutive treatment with 80 ppm in drinking water (intake of AA was 12.19 mg/kg/day) but not in 11-week-old adult rats (intake of AA was 7.05 mg/kg/day) [28]. Additionally, genotoxicity induced by 60 days of repeated treatment of approximately 1.4-mM AA in drinking water (intake of AA was 7.7–10.3 mg/kg bw/day) has been examined by a *cII* mutant assay in bone marrow using Big Blue F344 male and female rats, and it was demonstrated that AA effects were negative in male rats but slightly positive in female rats [27]. These findings suggest that erythroid precursors and/or HSCs in bone marrow can be target organs of AA genotoxicity, but the detection levels of AA genotoxicity may be dependent on differences in age and/or sex. Moreover, it was mentioned that AA genotoxicity analyzed by the RBC *Pig-a* assay and the high throughput protocol of *Pig-a* assay [43] was inconclusive (most likely negative) after 28 consecutive days of dosing at 20 mg/kg/day [39]. These findings are consistent with our results from single dosing experiments and suggest that it is difficult to detect AA genotoxicity in adult male rats by the RBC *Pig-a* and PIGRET assays.

We conclude that no clear AA genotoxicity was demonstrated by the results from the RBC *Pig-a* and PIGRET assays with a single dose of AA up to the LD50 to male adult rats. However, further analyses using rodents at different ages and sex with both acute and repeated dosing are needed to clarify whether the *Pig-a* assays can or cannot detect the AA-induced genotoxicity.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Measuring reproducibility of dose response data for the *Pig-a* assay using covariate benchmark dose analysis

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ABSTRACT

The reproducibility of the *in vivo Pig-a* gene mutation test system was assessed across 13 different Japanese laboratories. In each laboratory rats were exposed to the same dosing regimen of *N*-nitroso-*N*-ethylurea (ENU), and red blood cells (RBCs) and reticulocytes (RETs) were collected for mutant phenotypic analysis using flow cytometry. Mutant frequency dose response data were analysed using the PROAST benchmark dose (BMD) statistical package. Laboratory was used as a covariate during the analysis to allow all dose responses to be analysed at the same time, with conserved shape parameters. This approach has recently been shown to increase the precision of the BMD analysis, as well as providing a measure of equipotency. This measure of equipotency was used here to demonstrate a reasonable level of interlaboratory reproducibility. Increased reproducibility could have been achieved by increasing the number of cells scored, as this would reduce the number of zero values within the mutant frequency data. Overall, the interlaboratory trial was successful, and these findings support the transferability of the *in vivo Pig-a* gene mutation assay.

1. Introduction

During validation of the *in vivo Pig-a* gene mutation test system, 13 Japanese laboratories carried out a ring trial to test for assay reproducibility of the dose response following exposure to a mutagenic substance. This supports the previous international ring trial, in which the analytical techniques used to evaluate *Pig-a* mutation as well as the applied statistical approaches were different, but the test chemical was the same [1]. Dose responses were generated in red blood cells (RBCs) and reticulocytes (RETs) following exposure to the same set of *N*-nitroso-*N*-ethylurea (ENU) doses. Samples were taken at weeks 0, 1, 2 and 4, with week 4

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being the standard time for generating dose response data using this approach. The *Pig-a* assay is based on flow cytometric scoring, which allows for large numbers of events to be analysed in a quick and automated manner. Due to this ability to provide large data sets and the increased use of mutation data for quantitative purposes in human health risk assessment, it was considered appropriate to compare the dose responses and to test for reproducibility. The benchmark dose (BMD) approach has recently been championed for use in defining points of departure (PoD) for genetic toxicity endpoints [2–5], and more recently for defining potency ranks as a measure of equipotency [6,7].

Recent work conducted by researchers at the Dutch National Institute of Health and the Environment (RIVM) has shown that appropriate use of BMDs in context of their confidence limits has applications for compound potency ranking within an endpoint, as well as empirical potency comparisons across endpoints [8–11]. Furthermore, novel computational algorithms developed at the RIVM permit combining datasets for the same endpoint and analogous functional form. These algorithms enable simultaneous BMD analyses to be conducted across covariates (*e.g.*, compound, tissue, cell type, sex, exposure duration/regime, genotype *etc.*) and importantly have the potential to yield more precise BMD estimates where normalised response shape is conserved across covariates for a shared endpoint [12–15].

When comparing dose responses, it is essential that the data are represented on suitable axis, and there is not any bias placed on the data through any visual critiquing. This is achieved in the PROAST BMD analysis, through the assumption that biology is 'multiplicative' compared to being 'additive', which leads to a default log transformation of both axis. This transformation leads to analysis of fold changes compared to absolute changes in metrics, which are often not very comparable. Further assumptions are used when carrying out covariate BMD analysis, including each dose response within this series of experiments having conserved shape parameters for maximum response (c) and log-steepness (d), while parameters for background (a), potency (b) and *var* (*i.e.*, within group variation) were covariate dependant [15]. These key assumptions are based on a recent re-analysis of a large number of toxicological datasets indicating that the dose-responses for a given (continuous) endpoint from different chemicals tend to have similar shapes [15]. This approach has been tested and validated for use in potency ranking [6,7,15].

There are some major advantages when using the covariate approach, such as an increase in BMD precision, because certain dose response information is used from the other dose responses when fitting the model. Wills et al. (2016b) have shown that it can be of great benefit to include data from a study with many doses and replicates tested to improve the BMD estimate from a study with minimal data [7]. Along with increased precision, the discussion also moves away from whether the results are only positive or negative, to discussions about potency. Previous efforts to measure equipotency for genotoxicity endpoints have relied on metrics such as no observed genotoxic effect levels (NOGEL) or lowest effect dose (LED), however these are imprecise estimates of potency and are highly sensitive to experimental design differences, while they do not provide a measure of uncertainty [12,15]. The covariate BMD approach therefore provides a more suitable method for defining equipotency between different data sets, while providing further information as well.

The aim was to use the BMD covariate approach to rank the BMD metrics for *Pig-a* Mutant Frequency (MF) for each laboratory, to see whether the different laboratories produced BMD that were equivalent to each other.

2. Materials and methods

2.1. In vivo *pig-a* assay

Table 1 provides information on *Pig-a* study design of the different participant laboratories, with further details in the paper within this special issue [16].

2.2. *BMD covariate approach for potency ranking*

Pig-a dose response datasets were obtained from the different laboratories as stated above. These data were then subjected to combined BMD analyses through combination of dose-response relationships with laboratory as covariate. Data from red blood cells (RBC) and reticulocytes (RET) were analysed separately. *Pig-a* mutant frequency response at 4 weeks after treatment is more stable than other earlier time points and it is appropriate to perform a covariate BMD analysis among participant laboratories. As presented in Wills et al., historical dose-responses for the same endpoint but with a different chemical can be used to increase precision of the BMD estimate [7]. An extensive *Pig-a* MF data set containing 6 dose levels of alkylating agent methyl methanesulfonate [17] was therefore used to improve the BMD analysis in which 2 dose groups were tested for ENU. This approach allows any differences in BMDL-BMDU to be more clearly observed, by reducing the width of these BMD confidence intervals, as observed in Figs. 1 and 2, which include the Zeller et al. (2016) data, compared to Supplementary Fig. 5 which does not.

PROAST version 61.2 was used to conduct the dose-response analyses (http://www.proast.nl). Dose-response data were analysed using both the exponential and the Hill nested model families, as recommended by the European Food Safety Authority (EFSA) for the analysis of continuous data [18]. PROAST uses the likelihood ratio test to assess whether inclusion of additional parameters resulted in a statistically significant improvement in model fit [6,7,9,12,14]. Models with additional parameters are only accepted if the difference in log-likelihood exceeds the critical value at $p < 0.05$ [15]. In this way, it can be established which model parameters need to be estimated for each subgroup, and which parameters may be considered as constant among the subgroups of a combined dataset. In general, it was assumed that the maximum response (parameter *c*) and log-steepness (parameter *d*) (*i.e.*, shape parameters) were equal for all response curves, while parameters for background response (parameter *a*), potency (parameter *b*) and *var* (*i.e.*, within group variation) were covariate dependent [15]. PROAST outputs designate potency for each level of the covariate (*i.e.*, the BMD) as CED or Critical Effect Dose, and the metrics BMDL and BMDU are designated CEDL and CEDU, respectively. Fits of the model to the datasets of each subgroup are presented in the Supplementary figures, and were used to visually evaluate the (approximate) validity of the assumed constant shape parameters. This approach was preferred over evaluating the assumption by statistical testing, since statistical tests on the shape parameters are highly sensitive to non-random errors in the data that are ubiquitous in experimental data, and the effect of which may even be amplified by leverage effects in dose-response data [15]. Furthermore, minor non-random errors in the data might lead to rejection of the constancy of the shape parameter assumption (*i.e.*, given the relatively high power in a combined dataset), while small differences among the shape parameters would probably only have a small impact on the coverage of the BMD confidence interval [15]. Visual inspection of the fitted curves was therefore considered a better way to determine whether any differences in parameters *c*

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Table 1
Pig-a MF dose response data from each laboratory, tested at 4 weeks in RETs and RBCs. These data were used for each analysis. Data from Zeller et al. [17] not included. 'Response' refers to number of mutant cells per million wild-type cells.

Labs	Rat strain	Age (weeks)	ENU dose level (mg/kg)	ENU Lot #	Employed antibodies for Pig-a assays (RBC *Pig-a* assay and PIGRET assay)	Flow cytometer	Software
A	Crl:CD(SD)	8	0, 10, 40	SLBF6373V	HIS49-APC/CD71-PE/CD59-FITC	FACSCalibur	Cell Quest ver. 2.6
B	Crl:CD(SD)	8	0, 10, 40	SLBF6373V	HIS49-APC/CD71-PE/CD59-FITC	FACSCanto	FACSDiva ver. 4.1.2
D	Crl:CD(SD)	8	0, 10, 40	SLBF6373V	HIS49-APC/CD71-PE/CD59-FITC	FACSVerse	FACSuite ver 1.03
F	Slc:SD	8	0, 10, 40	SLBF6373V	HIS49-APC/CD71-PE/CD59-FITC	FACSCanto II	FACSDiva ver. 6.1
G	Crl:CD(SD)	8	0, 10, 40	SLBF6373V	HIS49-APC/CD71-PE/CD59-FITC	FC500 MPL	MXP Cytometer Ver 2.2
I	Crl:CD(SD)	8	0, 10, 40	SLBG0975V	HIS49-APC/CD71-PE/CD59-FITC	FACSCanto	FACSDiva ver. 4.1
J	RccHan:WIST	8	0, 10, 40	SLBF6373V	HIS49-APC/CD71-PE/CD59-FITC	FACSCanto II	FACSDiva ver. 6.1
K	Crl:CD(SD)	8	0, 10, 40	SLBF6373V	HIS49-APC/CD71-PE/CD59-FITC	FACSVerse	FACSuite ver.1.0.2.2238
L	Crl:CD(SD)	8	0, 10, 40	SLBD3983V	HIS49-PerCP/CD71-PE/CD59-FITC	Epics XL	XL SYSTEMII
M	Crl:CD(SD)	8	0, 10, 40	SLBF6373V	HIS49-APC/CD71-PE/CD59-FITC	FACSCantoII	FACSDiva ver. 6.1.2
N	Crl:CD(SD)	8	0, 10, 40	SLBF6373V	HIS49-APC/CD71-PE/CD59-FITC	FACSCalibur	Cell Quest Pro
O	Crl:CD(SD)	8	0, 10, 40	SLBG0975V	HIS49-APC/CD71-PE/CD59-FITC	FACSVerse	FACSuite ver. 1.05
P	Crl:CD(SD)	8	0, 10, 40	SLBF6373V	HIS49-APC/CD71-PE/CD59-FITC	FACSCanto II	FACSDiva ver. 6.1

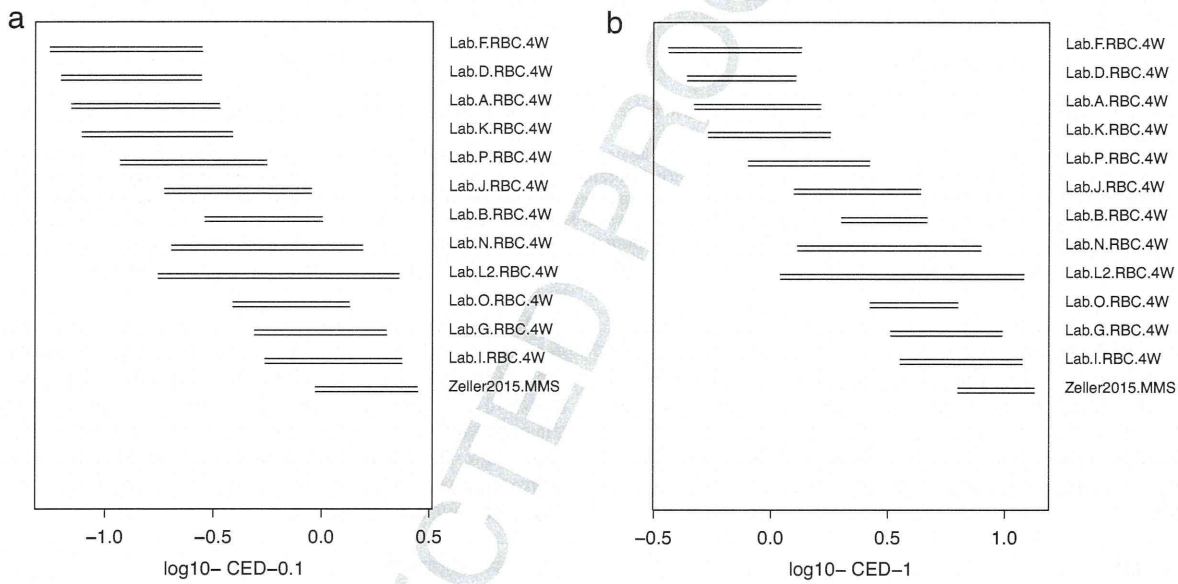

Fig. 1. RBC Week 4: *Pig-a* MF dose response data following exposure to ENU from the different laboratories was analysed using the BMD covariate approach, using BMRs of (a) 10% and (b) 100%.
One MMS dose response data set from Zeller et al. [17] was used to increase the precision of the BMD estimates [7]. The 4 parameter exponential (top horizontal lines) and Hill (bottom horizontal lines) models provided a suitable fit to the data, with 'laboratory' used as covariate. The width of the horizontal lines represents the BMDL-BMDU, which are ranked from lowest to highest concentration by BMD. During this combined analysis, the maximum response (parameter c) and log-steepness (d) parameters were assumed equal for all response curves, while parameters a (background response), b (potency) and *var* (within group variation) were covariate dependant. The use of constant 'shape' parameters (parameters c, d) still provided a strong description of the individual response curves. Overlapping lines show equipotency, with potency decreasing from top left to bottom right. Lab M did not produce RBC *Pig-a* MF. TOP: CED-0.1 is equivalent to, or another name for, BMD_{10}. BOTTOM: CED−1 = BMD_{100}. X-axis are Log10.dose(mg/kg/day); Y-axis are laboratory.

and *d* between covariates were small enough to be ignored. Residual errors and within-group variances were visually examined for compliance to log-normality and homogeneity, respectively.

The Bench Mark Response (BMR), also known as Critical Effect Size (CES in PROAST notation), employed in the presented analyses was set at 10%. This is justified since the aim of the analyses was to examine differences in potency rather than derive a point of departure for risk assessment. The BMDL and BMDU values represent the lower and upper bounds of the two-sided 90%-confidence interval of the BMD [14], with the BMDU-BMDL ratio defining the width of the confidence interval and therefore its precision. Confidence interval plots, arranged using the geometric midpoint of the BMDL-BMDU interval were employed to visually compare potencies across levels of examined covariates whilst taking estimation uncertainty into account [19].

3. Results

The 'maximal' (four-parameter) exponential model provided a suitable fit to the RBC and RET data at 4 weeks sampling time using PROAST (v61.2). The covariate BMD approach using constant shape parameters was used to generate Figs. 1 and 2, which show the $BMDL_{10}$-$BMDU_{10}$ and $BMDL_{100}$-$BMDU_{100}$ plots, ranked by the midpoints of the interval [19], for RBC and RET, respectively, with the laboratory on the X axis and $\log_{10}$ of concentration (mg/kg) on the Y axis. The supplementary figures show the dose response modelling for each of these data sets, and the Hill and exponential models provide suitable fits to the data. Supplementary Fig. 5 also shows the analyses carried out without data from Zeller et al. (2016), in which the $BMDL_{10}$-$BMDU_{10}$ are wider and less precise which leads to more overlap between laboratories. Figs. 1 and 2 show similar

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