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Compensatory erythropoiesis has no impact on the outcome of the in vivo Pig-a mutation assay in rats following treatment with the haemolytic agent 2-butoxyethanol

Michelle O. Kenyon*, Stephanie L. Coffing, Joel I. Ackerman, William C. Gunther, Stephen D. Dertinger1, Kay Criswell and Krista L. Dobo

Pfizer Worldwide Research and Development, Genetic Toxicology, Eastern Point Road, MS-8274-1317, Groton, CT 06340, USA and Litron Laboratories, 3500 Winton Place, Rochester, NY 14623, USA

*To whom correspondence should be addressed. Tel: +1 860 715 0643; Fax: +1 860 686 0548; Email: michelle.o.kenyon@pfizer.com

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Abstract

The Pig-a assay has rapidly gained international interest as a useful tool for assessing the mutagenic potential of compounds in vivo. Although a large number of compounds, including both mutagens and non-mutagens, have been tested in the rat Pig-a assay in haematopoietic cells, there is limited understanding of how perturbations in haematopoiesis affect assay performance. Of particular concern is the possibility that regenerative haematopoiesis alone, without exposure to a genotoxic agent, could result in elevated Pig-a mutant cell frequencies. To address this concern, Wistar-Han rats were dosed by oral gavage with a non-genotoxic haemolytic agent, 2-butoxyethanol (2-BE). Dose levels ranging from 0 to 450 mg/kg were tested using both single administration and 28-day treatment regimens. Haematology parameters were assessed at minimum within the first 24 h of treatment and 8 days after the final administration. Pig-a mutant frequencies were assessed on Days 15 and ~30 for both treatment protocols and also on Days 43 and 57 for the 28-day protocol. Even at doses of 2-BE that induced marked intravascular lysis and strong compensatory erythropoiesis, the average Pig-a mutant phenotype red blood cell and reticulocyte frequencies were within the historical vehicle control distribution. 2-BE therefore showed no evidence of in vivo mutagenicity in these studies. The data suggest that perturbations in haematopoiesis alone do not lead to an observation of increased mutant frequency in the Pig-a assay.

Introduction

The in vivo Pig-a assay, which identifies cells with a mutant Pig-a phenotype based on absence of glycosylphosphatidylinositol-anchored cell surface markers, was first introduced for use in rodents in 2008 (1–4). The assay has rapidly developed and gained interest due to its usefulness for assessing the mutagenic potential of compounds in vivo (5). This is evidenced by its adoption for development by the International Life Sciences Institute Health and Environmental Science Institute in 2012 and as a topic of the 2013 International Workshop on Genotoxicity Testing (IWGT).

Although a large number of compounds, including both mutagens and non-mutagens, have been tested in the rat Pig-a assay in haematopoietic cells, there is limited understanding of how toxicity to the haematopoietic system affects assay performance. Toxicity to red blood cells (RBCs) does not appear to affect the sensitivity of the Pig-a assay as many mutagenic compounds that result in reduction in the percentage of reticulocytes (Rets), including but not limited to N-ethyl-N-nitrosourea, ethyl methane sulphonate, N-methyl-N-nitrosourea, 7,12-dimethyl-1,2-benz[a]anthracene and aristococchic acids, have been correctly identified as mutagenic using the Pig-a assay (6–10). However, the effect of RBC toxicity on the spontaneous mutation rate in the Pig-a assay has not been reported. In fact, the IWGT Pig-a Workgroup identified the lack of understanding of the effect of stress erythropoiesis on Pig-a mutant frequencies as a validation data
gap (11). Haemolysis can induce compensatory or stress erythropoiesis, resulting in the regeneration of Rets (12). It is possible that clonal expansion of spontaneous mutants during this regeneration could result in an elevated Pig-a mutant frequency. On the other hand, Pig-a mutations are thought to be growth neutral (9,13,14). Therefore, selective expansion of mutants would not necessarily be expected since both mutants and wild-type cells should expand at a similar rate (15).

To test whether compensatory erythropoiesis could lead to expansion of Pig-a mutants and thereby misclassification of non-genotoxic agents as mutagens, peripheral erythrocytes were examined for the Pig-a mutant phenotype following exposure of Wistar-Han rats to 2-butoxyethanol (2-BE), a model non-genotoxic haemolytic agent (16–19). Both acute (single day) and repeat dosing (28-day) treatments were studied. The resulting data are discussed in terms of the potential of the Pig-a assay to evaluate compounds for mutagenic potential, whether or not they perturb erythropoiesis.

Materials and Methods

Chemicals and reagents
2-BE (CAS 111-76-2) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anticoagulant, balanced salt solution, anti-CD61 phycoerythrin (PE) solution, anti-CD59 PE solution and SYTO® 13 dye were supplied in the MutaFlow® Pig-a mutation assay kit purchased from Litron Laboratories (Rochester, NY, USA). Anti-PE paramagnetic beads and MACS® magnetic separation columns were purchased from Miltenyi Biotec (Auburn, CA, USA). CountBright counting beads were purchased from Invitrogen (Eugene, OR, USA). Lympholyte® mammal cell separation media was purchased from Cedarlane (Burlington, NC, USA) and was used for depletion of leukocytes and platelets. Heat-inactivated foetal bovine serum was purchased from Sigma.

Animals
Male Wistar-Han IGS [Crl: WI (Hans)] rats from Charles River Laboratories International, Inc. (Wilmington, MA, USA; ~180–200 g and 7–8 weeks old at the start of treatment) were used in all experiments. Certified Rodent Diet 5002 (PM® Nutrition International Certified LabDiet®, St. Louis, MO, USA) and municipal drinking water, further purified by reverse osmosis, were provided ad libitum. Animals were housed individually in polycarbonate boxes in a room with relative humidity of 50 ± 20%, temperature of 68–79°F and a 12-h light/dark cycle. Animal observations were made and body weights collected on Days 1, 15 and 33 in the acute study and once a week throughout the dosing phase in the sub-chronic study. All animal care and experimental procedures were conducted in compliance with the Pfizer's Institutional Animal Care and Use Committee.

Experimental design
Rats were randomly assigned to dose groups (6 animals/group) by generating a random list of animal numbers. 2-BE was dissolved in water and administered by oral gavage in a dose volume of 10 ml/kg. For the acute study, dosing formulations were prepared with water as the vehicle just prior to dose administration. In the sub-chronic study, dosing formulations were prepared with water as the vehicle 3 times/week and stored at 4°C. The formulations were stirred throughout dosing to ensure a homogenous dosing stock. In the acute study, animals received a single dose of 2-BE (10, 35, 100, 250 or 450 mg/kg) or vehicle. In the 28-day study, animals received a dose of 2-BE (10, 100, 250 or 450 mg/kg/day) or vehicle daily for 28 days.

Blood collection
Animals were anaesthetised by isoflurane inhalation prior to blood collection. All blood collection occurred via jugular venipuncture. Blood collection time points are shown in Table 1 for both the acute and 28-day treatments.

Haematology
Approximately, 500 µl of peripheral blood was collected in K$_2$EDTA tubes. Sample analysis was performed using an Advia® 120 Haematology System, Siemens Medical Solutions USA, Inc. (Malvern, PA, USA). Erythrocyte (RBC) counts were enumerated by measuring forward scatter (FSC) of the laser light source as it was deflected by RBCs. Ret counts were enumerated based on labelling with RNA-specific fluorescent dye contained in the Siemens Autoretic Reagent (Siemens Medical Solutions USA, Inc.). Ret counts were reported as absolute and relative values. Mean corpuscular volume (MCV) was determined by the direct measurement of cell size measured by laser light scatter during the enumeration of the RBCs in the sample. Haemoglobin concentration (Hgb) was measured using a modified cyanmethaemoglobin reaction. Haematocrit (Hct) was calculated based on the MCV and RBC counts (RBC × MCV/10). Historical reference ranges for the haematology parameters were developed from internal data generated from 2008 to 2010 and have been updated yearly since then with new data.

Pig-a assay
Approximately 200 µl of peripheral blood was collected in K$_2$EDTA tubes. About 80 µl of blood was transferred from the microtainer tube to a microcentrifuge tube containing 100 µl of anticoagulant and mixed. This sample was used for Pig-a mutant analysis and enumeration of Rets. The remainder of each sample was retained in the original collection tube at 4°C until analysis was complete.

Sample labelling, processing and flow analysis were conducted according to methods described by Dertinger et al. (20) with slight modifications (MutaFlow® PLUS Instruction Manual for Rat Prototype High Throughput Pig-a Mutation Analysis, version 120910). Briefly, on the day of blood collection, blood samples were depleted of leukocytes and platelets, washed and stored overnight at ~4°C. Cell labelling with anti-CD59 and anti-CD61 antibodies, sample processing and flow analysis, occurred ~24-h post blood collection. Cells were labelled with anti-CD 61 and anti-CD 59-PE antibodies and incubated with anti-PE magnetic beads. A subset of these samples was designated as pre-column samples, incubated with counting beads and nucleic acid dye and then analysed by flow cytometry. The remaining samples were run over a magnetic column to deplete the wild-type cells, incubated with counting beads and nucleic acid dye and then analysed by flow cytometry. These samples were designated as post-column samples. Both the pre-column and the post-column samples were analysed on a BD FACScanto™ Flow Cytometer (BD Biosciences, San Jose, CA, USA) equipped with an argon laser and FACSDiva software. Pre-column samples were acquired using a high fluidics rate with a FSC threshold for ~1 min or until at least 1000 counting beads were counted. Post-column samples were acquired

<table>
<thead>
<tr>
<th>Table 1. Blood collection time points</th>
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<tr>
<td>Treatment protocol Haematology and biochemistry Pig-a assay</td>
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<tr>
<td>Acute (single dose) Days 15 and 33</td>
</tr>
<tr>
<td>28 Daily doses Days 2 (~24-h post-dose), 8 and 35 Days 15, 29, 43 and 57</td>
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</table>
under a high fluids rate for ~3 min with a FSC threshold until the sample was nearly exhausted.

Statistical analysis
Statistical analysis was performed using SAS®, version 9.2 (SAS Institute Inc., Cary, NC, USA). Haematology, biochemistry and Pig-a mutation assay parameters were analysed for significant trends by performing a one-way analysis of variance (ANOVA) to compare treatment groups. Parameters were analysed separately at each time point. Haematology, biochemistry and Pig-a analysis percent Rets data were untransformed, while the number of mutant Rets and the number of mutant RBC data were log transformed (base 10). In order to avoid taking the log of zero, a small constant (0.1) was added to each measured value for the mutant frequencies. The analysis for significant trends was performed using a sequential trend test with linear contrasts from ANOVA. The trend test for both mutant Rets and mutant RBCs was one-sided, testing for increasing trend. The first of the sequential trend tests for haematology, biochemistry, Pig-a analysis and % Rets was two sided. Subsequent trend tests for these parameters were one-sided based on whether the results of the initial trend test showed an increasing or decreasing trend. A pair-wise comparison using the Dunnett’s t-test was also performed so that statistical significance would not be missed in the case of an inverted U-shaped dose-response curve. The trend test was used to determine statistical significance of a response in all cases in these experiments given the shape of the dose-response curve. In line with the IWGT recommendation (11), a positive response in the Pig-a mutation assay was defined as a statistically significant trend with the average mutant frequency outside of the historical control 95% tolerance interval of 0–1.746 CD59° Rets/10° Rets and 0.009–1.321 CD59° RBCs/10° RBCs.

Results
Acute treatment

Clinical signs
There was no difference in the mean percent body weight change between the groups throughout the study following acute treatment with 2-BE. No clinical signs were noted in any animals.

Haematology
The effect of a single dose of 2-BE on RBC number is depicted in Figure 1A. Treatments of 250 and 450 mg/kg 2-BE resulted in statistically significant RBC depression of ~24% within the first 24 h after treatment, while treatments between 10 and 100 mg/kg did not cause a significant effect. By Day 8, RBC frequencies were within the historical reference range for all 2-BE doses; however, a statistically significant decrease in RBCs persisted in the 250- and 450-mg/kg dose groups (~10% and 4% compared with controls, respectively).

Decreases in Hgb and Hct correlated with RBC decreases in rats treated with single doses of 2-BE (Figure 1B and C, respectively). Statistically significant decreases in both Hgb (24–25%) and Hct (15–20%) were observed within the first 24 h after treatment at doses of 250 and 450 mg/kg 2-BE. Additionally, in the first 24 h, there was a statistically significant decrease in Hgb (~7%) compared with the controls at 100 mg/kg 2-BE, but the Hgb concentration was within the historical reference range. By Day 8, both Hgb and Hct were within the historical reference range for all 2-BE dose groups; however, there remained a slight but statistically significant decrease in Hgb at 250- and 450-mg/kg doses (~4 and 2%, respectively) compared with controls.

The effect of single treatments of 2-BE on MCV is depicted in Figure 1D. 2-BE doses of 250 and 450 mg/kg were found to cause a statistically significant increase in MCV compared with the controls within the first 24 h after treatment, but the effect was only outside of the historical reference range at the high dose. By Day 8, the MCV was statistically elevated and outside of the historical reference range at both 250 and 450 mg/kg. The effect of a single dose of 2-BE on Ret frequencies is depicted in Figure 2A–C. Within 24 h after treatment with 250 and 450 mg/kg 2-BE, the number (Figure 2A) and percentage of Ret (Figure 2B) were already statistically elevated in comparison with the concurrent controls and outside of the historical reference range. Ret number and percentage of Ret continued to rise on Day 8 in rats treated with 250 and 450 mg/kg of 2-BE. Based on analyses of percentage of Ret included in the Pig-a analysis (Figure 2C), by Day 15 Ret frequencies were returning to normal, though still statistically elevated at doses of 100–450 mg/kg and frequencies had returned to control levels by Day 33.

Pig-a mutation analysis
The effect of single doses of 2-BE on percentage of Ret and Pig-a mutant phenotype Rets and erythrocytes is depicted in Table 2. Although there was a statistically significant increase in the percentage of Ret on Day 15 of doses of 100–450 mg/kg 2-BE, there was no effect on either mutant Ret or erythrocyte frequencies at any doses of 2-BE tested. On Day 33, the percentage of Ret had returned to normal and there was still no change in mutant frequencies.

28-Day treatment

Clinical signs
In the sub-chronic study, there were differences in mean percent body weight change between the 2-BE-treated groups and the vehicle group. On average, the animals in the 250-mg/kg/day group gained no weight over study Days 1–15. With the 450-mg/kg/day group, the rats were observed to lose weight over this same period. By Day 29, animals in the 250- and 450-mg/kg/day 2-BE dose groups showed a similar body weight change from baseline as the vehicle control group.

Haemoglobinuria was observed for three of the six animals at 100-mg/kg/day group and all animals in both the 250- and 450-mg/kg/day groups. The observation was first made ~24 h after the first dose and disappeared within a week.

Haematology
The effect of 28 daily treatments of 2-BE on RBC number is depicted in Figure 3A. Treatments of 100, 250 and 450 mg/kg/day 2-BE resulted in statistically significant RBC depression of ~12 to 44% within the first 24 h after treatment with the two higher doses resulting in RBC frequencies below the historical reference range. On Day 8, decreases in RBC counts were similar to that observed on Day 2. By Day 35 (7 days after the last dose of 2-BE), RBC numbers had increased such that all doses were within the historical reference range; however, there was still a statistically significant decrease compared with the controls at 250 and 450 mg/kg/day (~7 to 10%).

Decreases in Hgb concentration and Hct correlated with RBC decreases in rats treated with 28 daily doses of 2-BE (Figure 3B and C, respectively). Statistically significant decreases in both Hgb (13–45%) and Hct (11–40%) were observed within the first 24 h after treatment with doses of 100, 250 and 450 mg/kg/day 2-BE. Although there was still a statistically significant decrease in both Hgb (11–24%) and Hct (6–14%) on Day 8, only the Hgb concentration at the
A 450-mg/kg/day dose was also below the historical reference range. By Day 35, observed values for both Hgb and Hct had returned to normal.

The effect of 28 daily treatments of 2-BE on MCV in rats is depicted in Figure 3D. 2-BE doses of 250 and 450 mg/kg were found to cause a statistically significant increase in MCV compared with the concurrent controls within the first 24 h after treatment, but the values were within the historical reference range. By Day 8, the MCV was statistically elevated and outside of the historical reference range in rats administered 250 and 450 mg/kg 2-BE. On Day 35, MCV was again within the historical reference range for all dose groups; however, there was still a statistically significant increase over the concurrent controls in the two highest dose groups. The effect of 28 daily doses of 2-BE on Ret frequencies in rats is depicted in Figure 4A–C. Within 24 h after treatment with 100, 250 and 450 mg/kg 2-BE, the number (Figure 4A) and percentage of Rets (Figure 4B) were already increased statistically in comparison with the concurrent controls and outside of the historical reference range in rats administered 250 and 450 mg/kg. Both Ret number and percentage of Rets continued to rise in rats treated with 100-, 250- and 450-mg/kg/day 2-BE as indicated by statistically significant increases above the historical reference range at all three doses. On Days 15–29, based on data from the Pig-a analysis (Figure 4C), the percent Rets were returning to control values, though still statistically elevated at doses of 100–450 mg/kg. On Days 35 (haematology) and 43 (Pig-a analysis), only percent Rets in animals doses with 250 and 450 mg/kg/day remained significantly elevated compared with concurrent controls, though within the historical reference range. By Day 57 (Pig-a analysis), values for percent Rets were back to control levels in all dose groups.

**Pig-a mutation analysis**

The effect of 28 daily doses of 2-BE on percentage of Rets and Pig-a mutant phenotype Rets and erythrocytes in rats is presented in Table 3. There was a statistically significant increase in percentage of Rets on Days 15 and 29 in animals administered 100–450 mg/kg/day of 2-BE. On Day 43, there was a slight, but statistically significant decreasing trend in percentage of Rets compared with the controls at
doses of 250 and 450 mg/kg/day. Ret values were consistent with the concurrent control levels in all dose groups on Day 57. There was no increase in mutant Ret or erythrocyte frequency observed at any doses of 2-BE tested on Days 15, 29 or 43 or on mutant erythrocyte frequency on Day 57. Although there was a modest increasing trend in mutant Ret frequency in the 450-mg/kg/day group on Day 57, the mean value was within the historical control tolerance interval and therefore did not meet a priori criteria for a mutagenic effect.

**Table 2.** Average Ret percentage (%Rets) and Pig-a mutant frequencies on Days 15 and 33 from Wistar rats receiving acute oral doses of 2-BE (0, 10, 35, 100, 250 and 450 mg/kg)

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Day 15</th>
<th>Day 33</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>%Rets</td>
<td>Mean mutant Rets per 10^6</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>2.22±0.26</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>10</td>
<td>2.25±0.31</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>35</td>
<td>2.42±0.53</td>
<td>1.4±3.1</td>
</tr>
<tr>
<td>100</td>
<td>3.23±0.37**</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>250</td>
<td>3.63±0.27**</td>
<td>0.2±0.2</td>
</tr>
<tr>
<td>450</td>
<td>3.80±0.61**</td>
<td>0.2±0.3</td>
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<tr>
<td></td>
<td></td>
<td>1.80±0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.00±0.21</td>
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<tr>
<td></td>
<td></td>
<td>1.92±0.16</td>
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<tr>
<td></td>
<td></td>
<td>1.45±0.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.68±0.21</td>
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<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Cells analysed for Pig-a mutant phenotype include RBCs and Rets.
**Significant increasing trend at P < 0.01 by ANOVA with linear contrasts.
Individual rat data associated with the 450 mg/kg/day treatment are provided in Table 4.

### Discussion

The Pig-a mutation assay represents a potentially important tool for examining chemical mutagenicity in vivo. To date, many known mutagens and some non-mutagens have been studied using the Pig-a mutation assay in rat peripheral blood. Given the encouraging performance characteristics of the assay, it is likely that it will be utilised to study chemicals of unknown mutagenic potential in the near future. However, an outstanding question regarding the effect of perturbations of haematopoiesis on assay results and interpretation is important to address before the assay is routinely used to evaluate chemicals of less well-characterised genotoxicity. The Pig-a mutation assay data presented herein for acute and 28-day treatments with 2-BE begins to address this question and suggests that for non-genotoxic chemicals induction of regenerative erythropoiesis is unlikely to confound the results and interpretation of the Pig-a assay.

2-BE is known to cause regenerative haemolytic anaemia in rats (17–19,21–25). This study of 2-BE included both acute and sub-chronic dose regimens that resulted in haemolysis with subsequent Ret regeneration. In line with the weight of evidence from existing genotoxicity data for 2-BE (16), the compound did not show any evidence of mutagenicity in either acute or 28-day treatment protocols.

With acute administrations of 2-BE, haemolytic anaemia was induced in the first 24 h after treatment at doses of 250 and 450 mg/kg in this study. This was characterised by a decrease in the number of circulating erythrocytes, Hgb and Hct and an increase in aspartate aminotransferase (AST) levels (Supplementary data, available at Mutagenesis Online). The induction of regenerative erythropoiesis was already evident in the first 24 h after treatment based on increases in absolute Ret number and percent Rets. There was also a concomitant increase in MCV, consistent with the increase in Rets, immature erythrocytes that are inherently larger than mature RBCs (12). By Day 8, intravascular lysis had ceased as evidenced by normal AST values (Supplementary data, available at Mutagenesis Online) and a normal number of circulating RBCs, Hgb and Hct,
Figure 4. Absolute Ret number (A) and percent Rets (B) on Days 2 (within 24 h after treatment), 8 and 35 from Wistar rats receiving 28 oral doses of 2-BE (0, 10, 100, 250 and 450 mg/kg). Percent Rets from Pig-a analysis (C) on Days 15, 29, 43 and 57. Solid line represents maximum historical reference value. Statistically significant trend at *P < 0.05 or **P < 0.01 using ANOVA with linear contrasts.

though RBC number and Hgb were still significantly decreased compared with controls. Regenerative erythropoiesis was still significant on Day 8 based on the increasing Ret number and percentage of Rets, as well as the increased MCV. These observed changes in haematology parameters were consistent with prior studies in which rats received 2-BE acutely (17,23,26).

The first analysis of Pig-a mutant frequencies in RBCs and Rets of rats treated with single doses of 2-BE was performed on Day 15, which is when mutant Rets are often first observed in the Pig-a assay after acute treatments (27,28). At this time, there was still a significant increase in percent Rets in 2-BE-treated animals, but values were returning to normal. A final analysis of mutant frequency was performed on Day 33 after the effects of proliferative regeneration of Rets were no longer evident based on data collected during the Pig-a mutant frequency analysis. There was no evidence of increases in mutant frequencies in either Rets or RBCs at either analysis time despite the drastic increase in Ret regeneration on Day 8 stimulated by 2-BE treatment relative to the concurrent control.

In the sub-chronic study, haemolytic anaemia was also induced within 24 h of the first 2-BE administration in rats dosed with 250 and 450 mg/kg with generally similar effects on haematology and serum chemistry parameters as seen in the acute study. In this test, however, the changes observed were often greater than those seen in the acute study and blood was observed in the urine of rats treated with doses of 2-BE ranging from 100 to 450 mg/kg/day. Additionally, there were significant changes at 100 mg/kg for many of the haematology parameters though most remained within the historical reference range. As animals were bled prior to the second 2-BE dose, this cannot be explained by the sub-chronic treatment protocol. These changes may be due to minor differences in actual compound concentrations received by the animals in the two tests and/or due to normal variability between animals. For example, the negative control average for RBC frequency and Hct were slightly higher in the sub-chronic study on Day 2 than in the acute study, while the average RBC frequency and Hct for animals treated with 100-mg/kg 2-BE were similar in the two studies.

Despite continuous dosing in the sub-chronic study, RBC number, Hgb (except at 450 mg/kg/day) and Hct returned to normal levels by Day 8 with some significant differences in comparison with controls similar to what was observed at the same doses
Table 3. Average Ret percentage (%Rets) and Pig-a mutant frequencies on Days 15, 29, 43 and 57 from Wistar rats receiving 28 oral doses of 2-BE (0, 10, 100, 250 and 450 mg/kg/day).

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>%Rets</th>
<th>Mean mutant RBCs per 10³</th>
<th>%Rets</th>
<th>Mean mutant RBCs per 10³</th>
<th>%Rets</th>
<th>Mean mutant RBCs per 10³</th>
<th>%Rets</th>
<th>Mean mutant RBCs per 10³</th>
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Cells analysed for Pig-a mutant phenotype include RBCs and Rets. Statistically significant trend at *P < 0.05 or **P < 0.01 by ANOVA with linear contrasts.

Based on this study in which rats were dosed acutely or subchronically with the non-genotoxic haemolytic agent, 2-BE, significant regenerative haematopoiesis was not confirmed for Pig-a mutation assay results, particularly at the typical analysis time point (~Day 29). This is the first published study to address the question of the impact of RBC toxicity on Pig-a mutation assay results. Testing of additional compounds that perturb haematopoiesis and/or studying of other inducers of stress erythropoiesis (e.g. repeat bleeds) is necessary to confirm the results of this study. Finally, for future studies, it would be valuable to administered to animals only once in the acute study. As with the acute study, MCV values, indicating larger erythrocyte size, continued to rise to Day 8, indicating that although intravascular lysis was no longer occurring there was more of an effect on Ret regeneration with continued treatment. This was also evident with Ret parameters on Day 8, where increases were outside of the historical range even at 100 mg/kg/day and by greater increases in absolute Ret numbers in comparison with the increases observed with a single dose of 2-BE. However, despite the continuous treatments to Day 28, percent Ret values were decreasing on Days 15–29, though statistically elevated compared with the control and above the historical range. These results are consistent with a prior multi-dose study where rats receiving 1–12 daily doses of 125 mg/kg 2-BE developed tolerance to the haemolytic effects (22). It has been proposed that this tolerance is associated with older erythrocytes being more sensitive to the effects of 2-BE than the newly formed RBCs that are generated during treatment (29).

The first analysis of Pig-a mutant frequencies in RBCs and Rets of rats treated with 28 daily doses of 2-BE was performed on Day 15, which is approximately halfway through the treatments. At this time point, there was still a significant elevation of percent Rets, but there was no evidence of Pig-a mutations in either cell population. On Day 29, one day after the final dose of 2-BE, a second analysis of Pig-a mutant frequencies was performed. At this time, there was still a significant increase in percent Rets, but Ret numbers were approaching normal. Again, there was no evidence of increases in the Pig-a mutant phenotype. At the last two sampling times, Days 43 and 57, there was no longer any evidence of proliferative regeneration of Rets. In fact, at the highest two dose levels, Rets were moderately lower than mean vehicle control. As with the earlier sampling times, except for a marginal increasing trend in mutant phenotype Rets, there was no clear evidence of increased mutant cell frequencies resulting from 2-BE exposure.

Inspection of individual animal mutant phenotype cell frequencies (Table 4) reinforces the contention that the increasing trend at Day 57 is not convincing. For one, there was no consistent increase in mutant Ret frequency over time. Additionally, when an individual animal presented with an increased mutant Ret frequency, there was generally no increase in mutant RBCs at the next analysis time as would be expected based on the kinetics of erythropoiesis. Upon mutagen exposure, mutations in the Pig-a gene can occur in the stem cell population or newly formed Rets. Mutant Rets then quickly mature into mutant RBCs. Accordingly, as time progresses, mutant RBCs are expected to replace wild-type RBCs increasing the mutant RBC response (27,30). In fact, in a protracted exposure study at late sampling times, it is the mutant phenotype RBC frequency that should be considered the primary endpoint of genotoxicity. There are two reasons for this: the mutant Ret measurements reflect a very brief period of time compared with the erythrocyte-based values, and owing to the number of events evaluated, the mutant Ret determinations are made with considerably less precision relative to mutant RBC values.
include an analysis of Pig-a mutant frequency at the peak of erythrocyte regeneration, especially as rats efficiently remove abnormal erythrocytes from circulation (31). This would be helpful for understanding whether regenerative haematopoiesis could have an effect on Pig-a mutant frequency if the peak in regeneration occurs at or closer to the time of analysis. In the meantime, when compounds with unknown genotoxic and haemolytic effects are evaluated using the Pig-a assay, it might be useful to include haematology parameters to assess early effects on RBCs and Retks as it is possible that the most dramatic effect on erythropoiesis will occur prior to the first time point selected for Pig-a mutant frequency analysis. This adds further support to the concept that the Pig-a assay, and indeed any genotoxicity assay, benefits from integrated study designs that provide additional toxicity data that can be extremely valuable when interpreting results.

**Supplementary data**

*Supplementary data* are available at *Mutagenesis* Online.

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Conflict of interest statement: S.D.D. is an employee of Litron Laboratories, a company that holds patents covering flow cytometric methods for scoring GPI anchor-deficient erythrocytes and sells kits based on this technology (In Vivo MutaFlow®). No conflicts of interest declared by the other authors.

**References**


