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***In vivo* Pig-a and micronucleus study of the prototypical aneugen vinblastine sulfate**

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Abstract

The *Pig-a* assay is being used in regulatory studies to evaluate the potential of agents to induce somatic cell gene mutations and an OECD test guideline is under development. A working group involved with establishing the guideline recently noted that representative aneugenic agents had not been evaluated, and to help fill this data gap *Pig-a* mutant phenotype and micronucleated reticulocyte frequencies were measured in an integrated study design to assess the mutagenic and cytogenetic damage responses to vinblastine sulfate exposure. Male Sprague Dawley rats were treated for twenty-eight consecutive days with vinblastine dose levels from 0.0156 to 0.125 mg/kg/day. Micronucleated reticulocyte frequencies in peripheral blood were determined at Days 4 and 29, and mutant cell frequencies were determined at Days –4, 15, 29, and 46. Vinblastine affected reticulocyte frequencies, with reductions noted during the treatment phase and increases observed following cessation of treatment. Micronucleated reticulocyte frequencies were significantly elevated at Day 4 in the high dose group. Although a statistically significant increase in mutant reticulocyte frequencies were found for one dose group at a single time point (Day 46), it was not deemed biologically relevant because there was no analogous finding in mutant RBCs, it occurred at the lowest dose tested, and only 1 rat exceeded an upper bound tolerance interval established with historical negative control rats. Therefore, whereas micronucleus induction reflects vinblastine's well-established aneugenic effect on hematopoietic cells, the lack of a *Pig-a* response indicates that this tubulin-binding agent does not cause appreciable mutagenicity in this same cell type.

Keywords

Pig-a gene; mutation; aneugen; micronuclei; genotoxicity

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Author contributions

S.D.D., J.C.B. and J.T.M. primarily designed these studies. S.L.A., C.L. and D.K.T. executed various aspects of the experiments. S.D.D. primarily wrote the manuscript, with significant input from all authors.

Disclosure

S.L.A., C.L., D.K.T., J.C. and S.D.D. are employees of Litron Laboratories, and J.T.M. has been a paid consultant. Litron holds patents covering flow cytometric methods for scoring micronucleated reticulocytes and sells kits based on this technology (*In Vivo* MicroFlow®). Litron holds patents covering flow cytometric methods for scoring GPI anchor-deficient erythrocytes and sells kits based on this technology (*In Vivo* MutaFlow®).

Introduction

Lack of glycosylphosphatidylinositol (GPI) anchored protein(s) on the cell surface of hematopoietic cells can be used as a reporter of phosphatidylinositol glycan-class A (*Pig-a*) gene mutation [Araten *et al.*, 1999; Chen *et al.*, 2001; Bryce *et al.*, 2008; Miura *et al.*, 2008; Rondelli *et al.*, 2013]. Flow cytometric analysis, in conjunction with antibodies against GPI-anchored cell surface markers such as CD59, allows one to score the incidence of non-fluorescent mutant cells relative to fluorescent wild-type cells. To date, studies involving rodent models have mainly focused on circulating erythrocytes, as these cells are easily obtained in sufficient quantity *via* small volume blood draws [Gollapudi *et al.*, 2015]. The low blood volume requirement, the *Pig-a* assay's compatibility with commonly used rodent strains, and the relatively low cost of these studies relative to other test systems makes this assay particularly attractive for studies of somatic cell mutations [Dobrovolsky *et al.*, 2010; Schuler *et al.*, 2011].

The *in vivo* *Pig-a* assay has been evaluated in international trials comprised of industry, regulatory agencies, and academia, with important expertise and organizational support provided by the Health and Environmental Science Institute's Genetic Toxicology Technical Committee (HESI-GTTC), as well as the International Workshop on Genotoxicity Testing (IWGT) [Schuler *et al.*, 2011; Gollapudi *et al.*, 2015]. Data generated to date demonstrate that with proper training inter- and intra-laboratory reproducibility as well as intra-laboratory repeatability of erythrocyte-based *Pig-a* assays are very high [Dertinger *et al.*, 2011a; Kimoto *et al.*, 2013 and 2016; Gollapudi *et al.*, 2015; Johnson *et al.*, 2016; Raschke *et al.*, 2016]. Furthermore, experience to date with reference genotoxicants indicates that flow cytometric analysis is capable of detecting treatment-related increases in the frequencies of mutant phenotype cells in the context of both short-term as and more protracted exposure schedules [Dertinger *et al.*, 2012; Kimoto *et al.*, 2016].

Acceptance of the rodent *Pig-a in vivo* gene mutation assay for regulatory applications requires documentation of responses to genotoxic chemicals that have been shown to act by different mechanisms of action. In 2013 the IWGT *Pig-a* Workgroup called attention to the fact that to their knowledge aneugens had not been tested [Gollapudi *et al.*, 2015]. To address this gap we chose to study vinblastine sulfate (VB), a *vinca* alkaloid used to treat a variety of cancers. VB is regarded as a potent aneugen, and this is attributable to its tubulin-binding properties that interfere with microtubule assembly and consequently leads to malsegregation of chromosomes during cell division. The agent's genotoxic mode of action is therefore most clearly documented at the chromosome level, especially aneugenicity via chromosome lag and/or non-disjunction. For example multiple reports document that VB and other *vinca* alkaloids induce *in vivo* micronucleus formation in erythrocyte-based assays [Vanparys *et al.*, 1990; MacGregor *et al.*, 2006], whereas single strand breaks and other lesions detected in the Comet assay are negative [Recio *et al.*, 2010]. Furthermore, using an *in vitro* assay system (*tk*+/- mouse lymphoma assay) in conjunction with DNA sequencing as well as microsatellite markers and whole chromosome probes, Honma and colleagues observed that VB-induced trifluorothymidine-resistant colonies were the result of allelic loss and not the result of *tk* locus gene mutation [Honma *et al.*, 2001].

The studies described above suggest that an X-chromosome-residing reporter such as *Pig-a* will not reliably detect loss of gene function resulting from chromosome malsegregation-type events, whereas the micronucleus endpoint, conducted with cells of the same origin, should be responsive to VB's aneugenic mode of action. The experiments described herein were performed to directly test this hypothesis, and thereby contribute to a better understanding of the type(s) of genotoxic activities detected by rodent *Pig-a* assays.

Materials and Methods

Reagents

VB (CAS No. 143-67-9) was purchased from Sigma-Aldrich, St. Louis, MO. Reagents used for flow cytometric enumeration of mutant erythrocytes (RBC^{CD59-}) and mutant reticulocytes (RET^{CD59-}) were from Rat MutaFlow[®] Kits and included Anticoagulant Solution, Buffer Solution, Nucleic Acid Dye Solution (contains SYTO[®] 13), Anti-CD59-PE, and Anti-CD61-PE (Litron Laboratories, Rochester, NY). Reagents used for flow cytometric MN-RET scoring (Anticoagulant Solution, Buffer Solution, DNA Stain, Anti-CD71-FITC and Anti-CD61-PE Antibodies, RNase Solution, and Malaria Biostandards) were from *In Vivo* Rat MicroFlow[®] Kits (Litron Laboratories). Additional supplies included Lympholyte[®]-Mammal cell separation reagent from CedarLane, Burlington, NC; Anti-PE MicroBeads, LS Columns, and a QuadroMACS[™] Separator from Miltenyi Biotec, Bergisch Gladbach, Germany; and CountBright[™] Absolute Count Beads and fetal bovine serum (FBS) from Invitrogen, Carlsbad, CA.

Animals, Treatments, Blood Harvests

Experiments were conducted with the oversight of the University of Rochester's Institutional Animal Care and Use Committee. Male Sprague-Dawley rats were purchased from Charles River Laboratories, Wilmington, MA. Rodents were allowed to acclimate for approximately one week, and their age at the start of treatment was approximately 7 weeks. Water and food were available *ad libitum* throughout the acclimation and experimental periods. VB was prepared at 0.714 mg/mL in 0.9% saline and 1mL aliquots were frozen at -20°C until use. For each of 28 consecutive days of administration, one aliquot was thawed and added to 0.9% saline to prepare working solutions of 0, 0.003125, 0.00625, 0.0125, 0.025 mg/mL. Administration was by intraperitoneal injection at 5 mL/kg body weight/day, for final dose levels of 0, 0.015625, 0.03125, 0.06125, or 0.125 mg/kg/day. Dose levels were chosen based on an 8-day dose range-finding experiment. Even so, during the definitive study morbidity became evident by Day 18 and only 2 of 6 rats in the highest dose group were treated beyond Day 22 for the intended 28 days.

Peripheral blood was collected four days before the first administration (i.e., Day -4) and mutant cell frequencies in these pre-dose blood samples were used to exclude animals that exhibited unusually high spontaneous frequencies. Rats with RET^{CD59-} or RBC^{CD59-} frequencies above the following upper bound values were deselected from the study: > 2.9 RET^{CD59-} and/or > 2.9 RBC^{CD59-} per million. These limit values correspond to one-sided 95% tolerance intervals (alpha = 0.05) [Vardeman, 1992], and provide coverage for 99% of future results based on a historical control database that includes 756 individual rats. Once

sufficient numbers of animals were identified for the study, they were randomly assigned to treatment groups (n = 6 per group).

Peripheral blood samples were also collected on Days 4, 15, 29, and 46. In all cases, blood was obtained by nicking a lateral tail vein with a surgical blade after animals were warmed briefly under a heat lamp. Approximately 200 μ L of free-flowing blood were collected directly into heparinized capillary tubes (Fisher Scientific, CAT no. 22-260-950). For *Pig-a* analyses, 80 μ L of each blood sample were transferred to tubes containing 100 μ L kit-supplied heparin solution where they remained at room temperature for less than 2 hr until leukodepletion as described previously [Dertinger *et al.*, 2012]. For the MN-RET endpoint, 30 μ L of each whole blood sample were transferred to tubes containing 100 μ L kit-supplied heparin solution where they remained at room temperature for less than 2 hrs, after which they were fixed with ultracold methanol [Torous *et al.*, 2003].

Micronucleated Reticulocytes: Sample Preparation, Data Acquisition

MN-RET and reticulocyte (RET) frequencies were scored for Day 4 and 29 blood samples *via* flow cytometry according to the *In Vivo* Rat MicroFlow Kit manual, these procedures are described in detail previously [Torous *et al.*, 2003; Dertinger *et al.*, 2004]. MN-RET frequency measurements were based on the acquisition of approximately 20,000 CD71-positive RET per blood sample. Instrument setup and calibration was performed using kit-supplied biological standards (*P. berghei*-infected blood cells) [Tometsko *et al.*, 1993]. A BD FACSCalibur flow cytometer running CellQuest Pro v5.2 software was used for data acquisition and analysis.

Pig-a Mutation: Sample Preparation, Data Acquisition

RET^{CD59-} and RBC^{CD59-} frequencies were determined using Day -4, 15, 29, and 46 blood samples *via* immunomagnetic depletion of wild-type erythrocytes and flow cytometric analysis, as described previously [Dertinger *et al.*, 2011b, 2011c, 2012]. In addition to reducing analysis times to 4 minutes per sample, immunomagnetic depletion made it practical to evaluate many times more cells than is otherwise feasible. For instance on Day 29, an average of 201×10^6 RBCs and 5.72×10^6 RETs per sample were evaluated for the CD59-negative phenotype.

Pig-a sample labeling and washing steps utilized deep-well 96 well plates from Axygen Scientific (cat. no. P-DW-20-C) to facilitate more efficient parallel processing. Flow cytometric analyses were also conducted using 96 well plates (U-bottom, Corning, cat. no. 3799) and the BD High Throughput Sampler (HTS) provided automated, walk-away flow cytometric analysis. These variations are described in the Rat MutaFlow Instruction Manual, v140403 (www.litronlabs.com).

An Instrument Calibration Standard was generated on each day of data acquisition. These samples contained approximately 50% wild-type and 50% mutant-mimic erythrocytes, and as described previously, provided a means to rationally and consistently define the location of CD59-negative cells [Phonethepswath *et al.*, 2010]. A BD FACSCanto II flow cytometer running Diva v6.1.2 software was used for data acquisition and analysis.

Calculations, Statistical Analyses

The formulas used to calculate RBC^{CD59-} and RET^{CD59-} frequencies based on pre- and post-immunomagnetic column data are described in the MutaFlow manual (www.litronlabs.com). The frequency of RETs and MN-RET were initially calculated as percentages, and the incidence of mutant phenotype cells were converted to number per 10⁶ cells. All %RET, %MN-RET, mutant cell frequencies, averages, and standard error calculations were performed in Excel Office X for Mac® (Microsoft, Seattle, WA).

For statistical evaluations, %MN-RET and RBC^{CD59-} and RET^{CD59-} frequencies (number per 10⁶) were log(10) transformed. Since zero RET^{CD59-} values were occasionally observed, a 0.1 offset was added to each RET^{CD59-} per 10⁶ number prior to log transformation. Each time point was studied separately, where the effect of treatment on these transformed MN-RET, RBC^{CD59-}, and RET^{CD59-} data was compared to vehicle control using Dunnett's multiple comparison t-tests in the context of a one-way analysis of variance (ANOVA) model (JMP, v12.0.1, SAS Institute Inc., Cary, NC). Significance was evaluated at the 5% level using a one-tailed test for increases relative to vehicle control. These same analyses were performed with %RET data as well, however in these cases data were not log transformed, and the tests were two-tailed.

When Dunnett's test indicated a significant increase to %MN-RET, RBC^{CD59-}, or RET^{CD59-}, two additional analyses were conducted to assess the biological relevance of the finding. Specifically, for each endpoint and time point in question, the entire set of MN-RET, RBC^{CD59-} or RET^{CD59-} data were evaluated for a dose-related increase. This assessment was performed with JMP's ANOVA platform, and required a regression effect to be statistically significant using an alpha value of 0.025. Additionally, for any significant treatment group, individual animal's values were compared against the distribution of historical negative control data in order to evaluate the proportion of animals that exhibited elevated frequencies. As with the study inclusion criteria described above, the *Pig-a* upper bounds tolerance intervals equal $2.9 \text{ RET}^{\text{CD59-}} \times 10^{-6}$ and $2.9 \text{ RBC}^{\text{CD59-}} \times 10^{-6}$. The MN-RET upper bounds tolerance interval (i.e., 0.19%) was based on 212 individual Sprague Dawley rats, a one-sided test with 95% coverage, and an alpha value of 0.05.

Results

Exposure to VB for 28 consecutive days resulted in a reduction to weight gain for the high dose animals (Table I). VB toxicity also significantly reduced RET frequencies (Figure 1). Day 4 %RET, measured during the micronucleus analyses, were significantly reduced in the high dose animals. On Day 15 two high dose rats' RET frequencies were so low (0.05 and 0.39%) that they precluded estimation of RET^{CD59-} frequencies. Additional indications of bone marrow toxicity were observed on Day 46, when the 0.03125 and 0.125 mg/kg/day groups exhibited significantly lower and higher values relative to concurrent vehicle controls, respectively. In the case of the high dose group, this effect likely reflects compensatory erythropoiesis.

RET^{CD59-} frequencies are shown in Figure 2. When evaluated by pair-wise tests, only the low dose group at the Day 46 time point was significantly elevated relative to concurrent

vehicle controls. The Day 46 RET^{CD59-} data did not exhibit a significant positive trend ($p = 0.5338$). Furthermore, only 1 rat in this group of 6 exceeded the historical negative control distribution (Figure 3). Thus, the one slightly elevated RET^{CD59-} group mean was considered not to be biologically significant. No statistically significant increases in RBC^{CD59-} frequencies were observed (Figure 4).

VB increased the frequency of MN-RET at the Day 4 time point in the 0.125 mg/kg/day dose group (Figure 5). A positive trend test was evident ($p = 0.0032$), and the majority of high dose animals (4 of 6) exhibited %MN-RET that exceeded the upper bound 95% tolerance interval (see Figure 6). Day 29 MN-RET showed no statistically significant effect, likely due to the fact that treatment was discontinued in all but 2 high dose group rats beyond Day 22. The two animals that were treated for the intended 28 days exhibited slightly elevated %MN-RET (0.14 and 0.18% MN-RET), but this is difficult to interpret based on only two individual rats. For comparison, the vehicle control averaged 0.11% MN-RET, with SEM of 0.008 and a range of 0.09 to 0.13%.

Discussion

Rodent-based *Pig-a* mutation assays have been undergoing systematic validation on an international level, with promising results [Dertinger *et al.*, 2011a; Kimoto *et al.*, 2013 and 2016; Gollapudi *et al.*, 2015]. Among the important advances made, the compatibility of erythrocyte-based assays with several mammalian species has been demonstrated [Dobrovolsky *et al.*, 2009, 2011; Bhalli *et al.*, 2011a; Dertinger *et al.*, 2015; Labash *et al.*, 2016; Cao *et al.*, 2016; Olsen *et al.*, 2017]. The suitability of acute versus more protracted treatment schedules has been investigated [Dertinger *et al.*, 2012; Gollapudi *et al.*, 2015; Kimoto *et al.*, 2016]. The effect of sex on baseline and mutagen-induced frequencies was also considered [Labash *et al.*, 2015]. The assay has been successfully adapted to immortalized cells for *in vitro* screening purposes [Krüger *et al.*, 2015; Nicklas *et al.*, 2015; Rees *et al.*, 2017]. Importantly, DNA sequencing results support the phenotype-genotype basis of the assay [Komoto *et al.*, 2011; Byrne *et al.*, 2014; Revollo *et al.*, 2015; Krüger *et al.*, 2016]. Much progress has also been made in regard to the number and types of chemicals investigated in the *in vivo* *Pig-a* assay, including structurally related mutagen/non-mutagen pairs [Bemis *et al.*, 2015; Labash *et al.*, 2016], potent clastogens [Bhalli *et al.*, 2013a], and promutagens that must undergo metabolic activation to form DNA-reactive intermediates [Bhalli *et al.*, 2011b and 2013b; Shi *et al.*, 2011; Dertinger *et al.*, 2012; Avlasevich *et al.*, 2014; Stankowski *et al.*, 2015; Kikuzuki *et al.*, 2016; Koyama *et al.*, 2016].

While the expansion of chemical classes tested has occurred at an impressive rate, to our knowledge no tubulin-binding aneugens have been studied to date. While loss of the X-chromosome (where the *Pig-a* locus resides) could conceivably result in the GPI anchor-deficient phenotype, we deemed this unlikely for erythroid precursor and progenitor cells that must exhibit sustained self-renewal and terminal differentiation capacities to affect peripheral blood compartment mutant RET and RBC frequencies. This view is in agreement with the IWGT *Pig-a* Working Group which noted the assay may not be sensitive to aneugenicity since such events would likely lead to cell death and not mutation [Gollapudi *et*

al., 2015]. This study provides the needed data to establish the relative sensitivity of the *Pig-a* and micronucleus assays to a prototypical tubulin-binding aneugen.

In agreement with VB's known genotoxic mode of action, Day 4 MN-RET frequencies were significantly elevated. While a statistically significant increase in RET^{CD59-} was found for one dose group at a single time point, its biological relevance is not supported because there was no analogous finding in mutant RBCs, and it occurred only at the lowest dose tested. Also, only 1 rat exceeded an upper bound value established with historical negative control rats. Thus, while VB causes cytogenetic damage to hematopoietic cells *in vivo*, it does not appear to cause appreciable mutagenicity to this same cell type. These results lend further support the complementary nature of the micronucleus and *Pig-a* assays, two endpoints that are easily combined into the same study and that utilize a small volume of peripheral blood taken from a single collection.

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Figure 1.

Percent reticulocyte (%RET) values are shown for individual rats exposed to one of five dose levels of vinblastine. Data corresponding to blood samples harvested on Days 4, 15, 29, and 46 are shown in panels A, B, C and D, respectively. Panels C and D each show 4 open circle (○) data points that correspond to high dose group rats that were not exposed to vinblastine for the entire 28 days of treatment due to their moribund condition. Green horizontal lines are mean values. Two-tailed Dunnett's test results are shown to the far right of each graph, where statistically significant differences relative to the concurrent vehicle control group are shown by dose levels written in italicized black text as opposed to red text, and by grey circles as opposed to red circles. Circles' diameters represent 95% confidence intervals.

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Figure 3.

Historical control mutant reticulocyte (RET^{CD59-}) frequencies (no. mutant cells per million) are graphed for each of 756 male Sprague Dawley rats. These naïve control rats were the same strain and approximate age (7 weeks) used in the current study. One sided (upper bound) tolerance intervals were calculated from these data based on log(10) transformed data, 95% coverage, alpha 0.05. The resulting tolerance interval, back transformed for the purposes of this graphical representation, equals 2.9×10^{-6} Mut RET, and is illustrated in this graph as a dashed line. Individual rat's Mut RET data for the one statistically significant group (i.e., low dose, Day 46) from the current study are shown at the far right, as red triangles. Only one of these low dose group animals exceeded this upper bound tolerance interval.

Figure 4.

Mutant erythrocyte ($\text{RBC}^{\text{CD59-}}$) frequencies are shown for individual rats exposed to one of five dose levels of vinblastine. Note that $\text{RBC}^{\text{CD59-}}$ values (no. per million) have been $\log(10)$ transformed. Data corresponding to blood samples harvested on Days 15, 29, and 46 are shown in panels A, B and C, respectively. Panels B and C each show 4 open circle ($\circ$) data points that correspond to high dose group rats that were not exposed to vinblastine for the entire 28 days of treatment due to their moribund condition. Green horizontal lines are mean values. One-tailed Dunnett's test results are shown to the far right of each graph. Circles' diameters represent 95% confidence intervals. No statistically significant increases relative to the concurrent vehicle control group were observed.

Figure 5.

Micronucleated reticulocyte (MN-RET) frequencies are shown for individual rats exposed to one of five dose levels of vinblastine. Note that MN-RET values (%) have been $\log_{10}$ transformed. Data corresponding to blood samples harvested on Days 4 and 29 are shown in panels A and B, respectively. Panel B shows 4 open circle (○) data points that correspond to high dose group rats that were not exposed to vinblastine for the entire 28 days of treatment due to their moribund condition. Green horizontal lines are mean values. One-tailed Dunnett's test results are shown to the far right of each graph. Statistically significant increases relative to the concurrent vehicle control group are shown by dose levels written in italicized black text as opposed to red text, and by grey circles as opposed to red circles. Circles' diameters represent 95% confidence intervals.

Figure 6.

Historical control micronucleated reticulocyte (MN-RET) frequencies (%) are graphed for each of 212 male Sprague Dawley rats. These vehicle control rats were the same strain and approximate age (7 weeks) used in the current study. One sided (upper bound) tolerance intervals were calculated from these data based on $\log(10)$ transformed data, 95% coverage, alpha 0.05. The resulting tolerance interval, back transformed for the purposes of this graphical representation, equals 0.19% MN-RET, and is illustrated in this graph as a dashed line. Individual rat's MN-RET data for the one statistically significant group (i.e., high dose, day 4) from the current study are shown at the far right, as red triangles. The majority of the high dose group animals (4 of 6) exceeded this upper bound tolerance interval.

Table 1

Vinblastine Dose (mg/kg/day)	Mean Weight Gain, Day 1–29 (grams)	% of Control
0	146	100
0.015625	157	107
0.03125	140	96
0.0625	144	98
0.125 *	118	81

* These data only include the two rats that were treated for the intended 28-days.