Mutat Res Gen Tox En 831 (2018) 33–44

Contents lists available at ScienceDirect

Mutat Res Gen Tox En

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

A statistical approach for analyzing data from the *in vivo Pig-a* gene mutation assay

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ARTICLE INFO

Keywords:
In vivo Pig-a test
Linear model
Multiple contrasts

ABSTRACT

The *in vivo Pig-a* gene mutation assay serves to evaluate the genotoxic potential of chemicals. In the rat blood-based assay, the lack of CD59 on the surface of erythrocytes is quantified *via* fluorophore-labeled antibodies in conjunction with flow cytometric analysis to determine the frequency of *Pig-a* mutant phenotype cells. The assay has achieved regulatory relevance as it is suggested as an *in vivo* follow-up test for Ames mutagens in the recent ICH M7 [25] step 4 document. However, very little work exists regarding suitable statistical approaches for analyzing *Pig-a* data. In the current report, we present a statistical strategy based on a two factor model involving 'treatment' and 'time' incl. their interaction and a baseline covariate for log proportions to compare treatment and vehicle data per time point as well as in time. In doing so, multiple contrast tests allow us to discover time-related changes within and between treatment groups in addition to multiple treatment comparisons to a control group per single time point. We compare our proposed strategy with the results of classical Dunnett and Wilcoxon-Mann-Whitney tests using two data sets describing the mode of action of Chlorambucil and Glycidyl methacrylate both analyzed in a 28-day treatment schedule.

1. 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. [1], Chen et al. [11], Bryce et al. [8], Miura et al. [36], Rondelli et al. [43]). Fluorescent antibodies against GPI-anchored cell surface markers such as CD59, together with flow cytometric analysis, can be used to score the incidence of *Pig-a* mutant cells (non-fluorescent) relative to wild-type cells (fluorescent). Rodent studies have mainly focused on circulating erythrocytes, as these cells are easily obtained in sufficient quantity *via* small volume blood draws (Gollapudi et al. [23]). The low blood volume requirement, the *Pig-a* assay's compatibility with commonly used laboratory animal models, and the low cost of these studies relative to other test systems makes this assay particularly attractive for studies of somatic cell mutation (Dobrovolsky et al. [18], Schuler et al. [44]).

Rodent-based *Pig-a* mutation assays have been undergoing systematic validation on an international level, with promising results (Dertinger et al. [12], Kimoto et al. [29] and [30], Gollapudi et al. [23]). For example the compatibility of erythrocyte-based assays with several mammalian species has been demonstrated (Dobrovolsky et al. [17,19], Bhalli et al. [4], Dertinger et al. [15], Labash et al. [35], Cao et al. [10], Olsen et al. [39]). The suitability of acute treatment schedules has been shown (Dertinger et al. [14], Gollapudi et al. [23], Kimoto et al. [30]). The effect of sex on baseline and genotoxicant-induced frequencies was also studied (Labash et al. [34]). The assay has been successfully adapted to immortalized cells for *in vitro* screening purposes (Krüger et al. [32], Nicklas et al. [37], Rees et al. [41]). Importantly, DNA sequencing results support the basic assumption that the phenotype measured by the assay is caused by *Pig-a* mutation (Kimoto et al. [28], Byrne et al. [9], Revollo et al. [42], Krüger et al. [33]). 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. [3], Labash et al. [35]), potent clastogens (Bhalli et al. [6]), and promutagens that require bioactivation to form DNA-reactive intermediates (Bhalli et al. [5] and [7]; Shi et al. [45], Dertinger et al. [14], Avlasevich et al. [2],

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https://doi.org/10.1016/j.mrgentox.2018.05.007
Received 4 January 2018; Received in revised form 29 March 2018; Accepted 4 May 2018
Available online 05 May 2018
1383-5718/

Stankowski et al. [46], Kikuzuki et al. [27], Koyama et al. [31]).

From above, it is clear that numerous aspects of the validation process are occurring at an impressive rate. However, to our knowledge, no systematic work has been conducted to determine the most appropriate statistical analyses. Whereas in 2013 the International Workshop on Genotoxicity Testing (IWGT) Pig-a Workgroup provided some general guidance (Gollapudi et al. [23]), there is a need for more systematic and detailed work in this area. A case can be made that this is especially important for the *Pig-a* assay, since it has several characteristics that combine to make it unusual among genetic toxicology endpoints: spontaneous frequencies are extremely low; mutagen-induced responses can exhibit a very large dynamic range; both early and late-responding cell cohorts are evaluated (reticulocytes and erythrocytes, respectively); and it is common practice to acquire longitudinal data from serial blood draws. The work reported herein especially focuses on this last aspect, the effective use of longitudinal data with the aid of an appropriate statistical model.

2. Material and methods

2.1. Experimental design

In recent years a protocol for the *Pig-a* assay has been established that is supported by inter-laboratory studies and by increasing the number of test substances, see Dertinger and Heflich [13], Schuler et al. [44] and Gollapudi et al. [23].

The standard design is a balanced randomized one-way layout using a vehicle/negative control plus at least 3 dose levels of the test substance; a positive control is not mandatory. In general, the animal is the experimental unit and animals are allocated randomly to treatment groups such that each group consists of a minimum of 5 animals from which data can be analyzed.

A treatment duration of 28 consecutive days is often preferred, as it allows the assay to be integrated into standard toxicology protocols (pivotal 4-week toxicity study). Usually, measurements are performed at different time points, for example before treatment starts ("Day −1"), in the middle of the treatment period ("Day 15"), at the end of the treatment phase ("Day 29"), and often include a post-treatment observation period ("recovery phase", *e.g.* "Day 43" or later) (see Fig. 1):

In this paper, our focus is on a design that involves a baseline measurement (prior to administration) and multiple (repeated) measurements after beginning the treatment protocol.

2.2. Sample preparation and data acquisition

The test chemicals considered in this study, along with their CAS No., vehicle, IARC classification, and dose levels, are provided in Table 1. Both test chemicals were purchased from Sigma-Aldrich, St. Louis, MO. Reagents used for flow cytometric CD59-negative erythrocyte (RBC^{CD59-}) and reticulocyte (RET^{CD59-}) scoring indicating Pig-a mutant genotype (Anticoagulant Solution, Buffered Salt Solution, Nucleic Acid Dye Solution [contains SYTO® 13], Anti-CD59-PE, and Anti-CD61-PE) were from Rat MutaFlow® Kits, Litron Laboratories, Rochester, NY. 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 from Invitrogen, Carlsbad, CA.

2.3. Animals, treatments, blood harvests

The procedures involving animals were approved by the Institutional Animal Care and Use Committees at the University of Rochester and the U.S. Food and Drug Administration/National Center for Toxicological Research (NCTR). Male Sprague-Dawley rats (chlorambucil study) were purchased from Charles River Laboratories, Wilmington, MA, USA; male F344 rats (glycidyl methacrylate study) were provided by an in-house breeding colony at the NCTR. Rodents were allowed to acclimate for approximately 1 week, and their age at the start of treatment was 7 weeks. Water and food were available *ad libitum* throughout the acclimation and experimental periods. As recommended by Gollapudi et al. [23], each treatment group consisted of 6 rats that were randomly allocated prior to substance application. Formulations of test substances were freshly prepared at the day of treatment and administered to rats *via* oral gavage in a volume of 10 mL/kg body weight/administration. Top-dose levels were based on preliminary dose-range finding experiments. As described in the OECD [38] Guideline for the Testing of Chemicals, No. 407 OECD [38], the aim was to find a top-dose level that induced toxic effects but not death or severe morbidity. Exposures occurred once a day at approximately 24 h intervals for 28 consecutive days.

Peripheral blood was collected before the first administration of test agent (*i.e.* at Day −1) with serial blood sampling on Days 15, 29 and 43 (chlorambucil study) or 56 (glycidyl methacrylate study), respectively. Blood was obtained by following the recommendations from the kit manufacturer.

2.4. Pig-a assay protocol

Pig-a analyses were in accordance with the Rat Blood MutaFlow® PLUS kit manual, v140403 (available at www.litronlabs.com; also see Dertinger et al. [14]). Each analysis was based on 80 µL of anticoagulant-treated blood. "Pre-column" samples consisted of a small aliquot of each fully labeled and stained sample which was analyzed for 1 min to provide %RET measurements as well as RBC or RET to Counting Bead ratios. The majority of each sample was then used for an immunomagnetic separation procedure, and the resulting "post-column" eluates were analyzed for 3 min to provide mutant phenotype RBC and mutant phenotype RET to Counting Bead ratios. As described previously, pre- and post-column data were used to calculate mutant phenotype RBC (RBC^{CD59-}) and mutant phenotype RET (RET^{CD59-}) frequencies (Dertinger et al. [14]). For the experiments described here, a typical sample provided $\geq 3 \times 10^6$ RET and $\geq 150 \times 10^6$ RBC equivalents per replicate. Instrument calibration and data acquisition were performed as described in the kit's instructions.

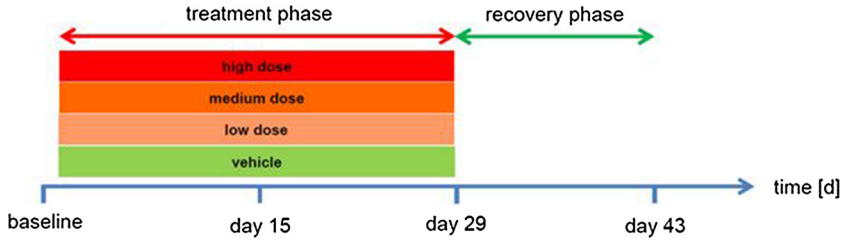

Fig. 1. Experimental design: usual 28-day study (+2 weeks recovery phase) incl. 1 vehicle and several test chemical dose groups.