Title: Measuring Reproducibility of Dose Response Data for the *Pig-a* Assay using Covariate Benchmark Dose Analysis

Author: George E. Johnson Mika Yamamoto Yuta Suzuki Hideki Adachi Takahiro Kyoya Hironao Takasawa Katsuyoshi Horibata Eri Tsutsumi Kunio Wada Ryuta Kikuzuki Ikuma Yoshida Takafumi Kimoto Akihisa Maeda Kazunori Narumi

PII: S1383-5718(16)30101-2
DOI: http://dx.doi.org/doi:10.1016/j.mrgentox.2016.04.004
Reference: MUTGEN 402728

To appear in: *Mutation Research*

Received date: 8-4-2016
Accepted date: 11-4-2016

Please cite this article as: George E.Johnson, Mika Yamamoto, Yuta Suzuki, Hideki Adachi, Takahiro Kyoya, Hironao Takasawa, Katsuyoshi Horibata, Eri Tsutsumi, Kunio Wada, Ryuta Kikuzuki, Ikuma Yoshida, Takafumi Kimoto, Akihisa Maeda, Kazunori Narumi, Measuring Reproducibility of Dose Response Data for the Pig-a Assay using Covariate Benchmark Dose Analysis, Mutation Research/Genetic Toxicology and Environmental Mutagenesis http://dx.doi.org/10.1016/j.mrgentox.2016.04.004

Measuring Reproducibility of Dose Response Data for the *Pig-a* Assay using Covariate Benchmark Dose Analysis

George E. Johnson[1], Mika Yamamoto[2], Yuta Suzuki[3], Hideki Adachi[4], Takahiro Kyoya[5], Hironao Takasawa[6], Katsuyoshi Horibata[7], Eri Tsutsumi[8], Kunio Wada[9], Ryuta Kikuzuki[10], Ikuma Yoshida[11], Takafumi Kimoto[12], Akihisa Maeda[13], Kazunori Narumi[14]

[1]Swansea University Medical School, Swansea University, SA2 8PP

[2]Drug Development Toxicology, Drug Safety Research Laboratories, Drug Discovery Research Division, Astellas Pharma Inc., 2-1-6, Kashima, Yodogawa-ku, Osaka, 532-8514, Japan

[3]Gotemba Laboratory, BoZo Research Center Inc., Gotemba-shi, Shizuoka 412-0039, Japan

[4]Preclinical Research Laboratories, Sumitomo Dainippon Pharma Co., Ltd., 3-1-98 Kasugadenaka, Konohana-ku, Osaka 554-0022, Japan

[5]Toxicology Laboratory, Life Science Research Institute, Kumiai Chemical Industry, Co., Ltd., 3360 Kamo, Kikugawa-shi, Shizuoka, 439-0031, Japan

[6]Safety Assessment Department, Nonclinical Research Center, Drug Development Service Segment, LSI Medience Corporation, 14-1 Sunayama, Kamisu-shi, Ibaraki 314-0255, Japan

[7]Division of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

[8]Quality Assurance Division, Safety Science Institute, Suntory Business Expert Limited, 8-1-1 Seikadai, Seika-cho, Soraku-gun, Kyoto 619-0284, Japan

[9]Toxicology Division, The Institute of Environmental Toxicology, 4321, Uchimoriya-machi, Joso-shi, Ibaraki 303-0043, Japan

[10]Drug Safety and Pharmacokinetics Laboratories, Taisho Pharmaceutical Co., Ltd., 1-403, Yoshino-cho, Kita-ku, Saitama-shi, 331-9530, Japan

[11]Drug Safety Research Laboratories, Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, 26-1, Muraoka-Higashi 2-chome, Fujisawa, Kanagawa, 251-8555, Japan

[12]Pharmaceutical Development Research Laboratories, Teijin Institute for Bio-medical Research, Teijin Pharma Limited, 4-3-2 Asahigaoka, Hino-shi, Tokyo 191-8512, Japan

[13]Toxicology and Pharmacokinetics Laboratories, Pharmaceutical Research Laboratories, Toray Industries Inc., 6-10-1 Tebiro, Kamakura, Kanagawa 248-8555, Japan

[14]Yakult Honsha Co., Ltd., 5-11 Izumi, Kunitachi-shi, Tokyo 186-8650, Japan

Highlights:

- Interlaboratory reproducibility is reasonable for in vivo *Pig-a* MF
- Covariate benchmark dose analysis provided a measure of reproducibility
- RBCs provided a greater level of *Pig-a* MF reproducibility and precision than RETs
- Reducing the number of zero values would increase overall reproducibility

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.

Key words:
OECD
Pig-a
Benchmark dose
Potency
In vivo
Dose response
Ring trial

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 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 axes, 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 axes. 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.

Materials and Methods

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].

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 2016, 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 Figures 1 and 2, which include the Zeller et al (2016) data, compared to Supplementary Figure 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* 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].

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 Figures 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 Figure 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 results, although in three datasets (labs) the confidence intervals related to the BMD for RET were relatively large (probably due to all observations in the controls being zero, i.e. below limit of quantification, LOQ). In both figures the confidence intervals do not overlap among all labs (datasets), indicating differences among some labs. Moreover, based on visual inspection of the figures, these differences are roughly estimated to be within only one order of magnitude.

Discussion:

The BMD potency ranking plots provide information on the reproducibility of the *Pig-a* dose response data. The overlapping confidence intervals established from mutant RBC and RET frequencies at week 4 show evidence that the mutant phenotype population can be reasonably well reproduced in different datasets, i.e. within one order of magnitude. Although the differences may be due to the labs, they could also just be replication error. This could not be established here, as no replicate studies within labs were available.

This is the first instance where potency estimates using BMD covariate analysis has been used to examine interlaboratory reproducibility. Non-overlapping BMD metrics and relatively large differences between the potential values of the true BMDs would indicate that the level of reproducibility was low. In the present ring study, we found reasonable reproducibility, but it would be worthwhile to improve it. This could be achieved by increasing the number of cells scored to a minimum of $1\text{-}5x10^6$ RETs or RBCs per animal, as this would reduce the number of zero's within the data [20]. In this regard, laboratory A was the only laboratory not to contain zero data points in the control *Pig-a* replicates of RETs, and laboratory A also has the smallest BMDL-BMDU width. For the RBC *Pig-a* MF, the laboratories that did not contain any zero's in control were B, G, L2 and O, and the BMDL-BMDU for each of these laboratories overlaps very well with each other (Figure 1).

Another example of the BMD covariate analysis approach in genetic toxicology is provided in the recent paper by Wills et al 2016, who found no significant differences between BMD potency estimates in different experimental replicates [7]. As another example the approach was used to show the effect of sampling day in *in vivo Pig-a* data. The approach is robust and provides a suitable way of comparing potencies across covariates (e.g. laboratories, sampling day, compound, sex, species etc.).

Acknowledgements: The authors would like to thank Dr John Wills and Dr Wout Slob for their expert input during preparation of the manuscript. We thank the ILSI/HESI Genetic Toxicology Technical Committee (GTTC) for initiating and supporting this collaboration.

References:

[1] S.D. Dertinger, S. Phonethepswath, P. Weller, J. Nicolette, J. Murray, P. Sonders, H.W. Vohr, J. Shi, L. Krsmanovic, C. Gleason, L. Custer, A. Henwood, K. Sweder, L.F. Stankowski, Jr., D.J. Roberts, A. Giddings, J. Kenny, A.M. Lynch, C. Defrain, F. Nesslany, B.J. van der Leede, T. Van Doninck, A. Schuermans, K. Tanaka, Y. Hiwata, O. Tajima, E. Wilde, A. Elhajouji, W.C. Gunther, C.J. Thiffeault, T.J. Shutsky, R.D. Fiedler, T. Kimoto, J.A. Bhalli, R.H. Heflich, J.T. MacGregor, International *Pig-a* gene mutation assay trial: evaluation of transferability across 14 laboratories, Environ. Mol. Mutagen. 52 (2011) 690-698.

[2] B.B. Gollapudi, G.E. Johnson, L.G. Hernandez, L.H. Pottenger, K.L. Dearfield, A.M. Jeffrey, E. Julien, J.H. Kim, D.P. Lovell, J.T. Macgregor, M.M. Moore, J. van Benthem, P.A. White, E. Zeiger, V. Thybaud, Quantitative approaches for assessing dose-response relationships in genetic toxicology studies, Environ. Mol. Mutagen. 54 (2013) 8-18.

[3] G.E. Johnson, L.G. Soeteman-Hernandez, B.B. Gollapudi, O.G. Bodger, K.L. Dearfield, R.H. Heflich, J.G. Hixon, D.P. Lovell, J.T. MacGregor, L.H. Pottenger, C.M. Thompson, L. Abraham, V. Thybaud, J.Y. Tanir, E. Zeiger, J. van Benthem, P.A. White, Derivation of point of departure (PoD) estimates in genetic toxicology studies and their potential applications in risk assessment, Environ. Mol. Mutagen. 55 (2014) 609-623.

[4] J.T. MacGregor, R. Frötschl, P.A. White, K.S. Crump, D.A. Eastmond, S. Fukushima, M. Guérard, M. Hayashi, L.G. Soeteman-Hernandez, G.E. Johnson, T. Kasamatsu, D. Levy, T. Morita, L. Müller, R. Schoeny, M.J. Schuler, V. Thybaud, IWGT Report on Quantitative Approaches to Genotoxicity Risk Assessment II. Use of Point-of-Departure (PoD) metrics in defining acceptable exposure limits and assessing human risk, Mutat. Res. 783 (2015) 66-78.

[5] J.T. MacGregor, R. Frötschl, P.A. White, K.S. Crump, D.A. Eastmond, S. Fukushima, M. Guérard, M. Hayashi, L.G. Soeteman-Hernandez, T. Kasamatsu, D. Levy, T. Morita, L. Müller, R. Schoeny, M.J. Schuler, V. Thybaud, G.E. Johnson, IWGT Report on Quantitative Approaches to Genotoxicity Risk Assessment I. Methods and metrics for defining exposure-response relationships and points of departure (PoDs), Mutat. Res. 783 (2015) 55-65.

[6] J.W. Wills, G.E. Johnson, S.H. Doak, L.G. Soeteman-Hernandez, W. Slob, P.A. White, Empirical analysis of BMD metrics in genetic toxicology part I: *in vitro* analyses to provide robust potency rankings and support MOA determinations, Mutagenesis, 31 (2016).

[7] J.W. Wills, A.S. Long, G.E. Johnson, J.C. Bemis, S.D. Dertinger, W. Slob, P.A. White, Empirical Analysis of BMD Metrics in Genetic Toxicology Part II: *In Vivo* Potency Comparisons to Enhance the Utility of Experimental Animals for Genetic Toxicity Assessment, Mutagenesis, 31 (2016).

[8] L.G. Hernández, J. Van Benthem, W. Slob, Estimating the carcinogenic potency of chemicals from the *in vivo* micronucleus test: RIVM Report 340700007/2012 RIVM Report (2012).

[9] L.G. Hernández, W. Slob, H. van Steeg, J. van Benthem, Can carcinogenic potency be predicted from *in vivo* genotoxicity data? a meta-analysis of historical data, Environ. Mol. Mutagen. 52 (2011) 518-528.

[10] L.G. Hernández, G.E. Johnson, L.H. Pottenger, J. van Benthem, Analysis of Low-Dose Mutagenic Responses and the Applicability of Genotoxicity Tests for Carcinogen Potency Prediction, Environ. Mol. Mutagen. 52 (2011) S26-S26.

[11] L.G. Hernández, W. Slob, H. van Steeg, J. van Benthem, Comparison of Carcinogenic Potency Estimates to *In Vivo* Genotoxic Potencies from the Micronucleus, Transgenic Rodent Mutation and Comet Assay Using the Benchmark Dose Approach, Environ. Mol. Mutagen. 51 (2010) 707-707.

[12] L. Soeteman-Hernandez, M. Fellows, G.E. Johnson, W. Slob, Carcinogenic potency estimation from the *in vitro* micronucleus test in TK6 cells: A pilot study, Toxicol. Sci. DOI: 10.1093/toxsci/kfv189 (2015).

[13] G.E. Johnson, W. Slob, S.H. Doak, M.D. Fellows, B.B. Gollapudi, R.H. Heflich, B.J. Rees, L.G. Soeteman-Hernandez, J.R. Verma, J. Wills, G.J.S. Jenkins, P.A. White, New Approaches to Advance the use of Genetic Toxicology Analyses for Human Health Risk Assessment, Toxicol. Res. 4 (2015) 667-676.

[14] L.G. Soeteman-Hernandez, G.E. Johnson, W. Slob, Estimating the carcinogenic potency of chemicals from the *in vivo* micronucleus test, Mutagenesis, DOI 10.1093/mutage/gev043 (2015).

[15] W. Slob, R.W. Setzer, Shape and steepness of toxicological dose-response relationships of continuous endpoints, Crit. Rev. Toxicol. 44 (2014) 270-297.

[16] D. Miura, e. al, Potential value of the *Pig-a* gene mutation assay in reticulocytes (PIGRET Assay) using rats as a short-term genotoxicity test: Summary of the collaborative study across 16 laboratories using 24 chemicals by the Mammalian Mutagenicity Study (MMS) Group of the Japanese Environmental Mutagenicity Society (JEMS), Mutat. Res. in this issue.

[17] A. Zeller, L. Tang, S.D. Dertinger, J. Funk, G. Duran-Pacheco, M. Guerard, A proposal for a novel rationale for critical effect size in dose-response analysis based on a multi-endpoint *in vivo* study with methyl methanesulfonate, Mutagenesis, 31 (2016).

[18] EFSA, Use of benchmark dose approach in risk assessment: Guidance of the Scientific Committee, 2009, 1150 (2009) 1-72.

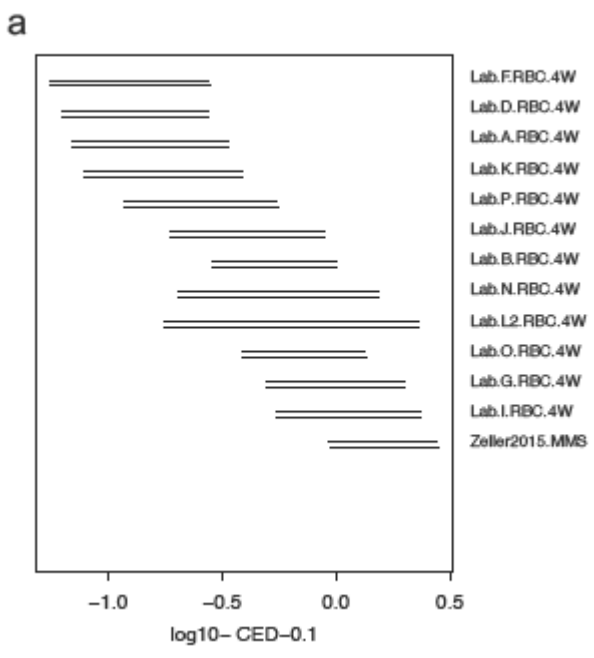
[19] J.C. Bemis, J.W. Wills, S.M. Bryce, D.K. Torous, S.D. Dertinger, W. Slob, Comparison of *in vitro* and *in vivo* clastogenic potency based on benchmark dose analysis of flow cytometric micronucleus data, Mutagenesis, 31 (2016).

[20] B.B. Gollapudi, A.M. Lynch, R.H. Heflich, S.D. Dertinger, V.N. Dobrovolsky, R. Froetschl, K. Horibata, M.O. Kenyon, T. Kimoto, D.P. Lovell, L.F. Stankowski Jr, P.A. White, K.L. Witt, J.Y. Tanir, The *in vivo Pig-a* assay: A report of the

International Workshop On Genotoxicity Testing (IWGT) Workgroup, Mutat. Res. 783 (2015) 23-35.

Figure Legends:

Figure 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 2016 [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-axes are Log10.dose(mg/kg/day); Y-axes are laboratory.



b

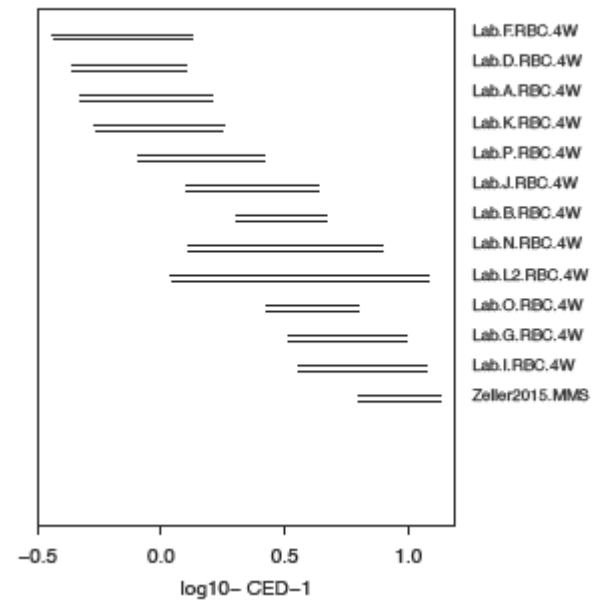
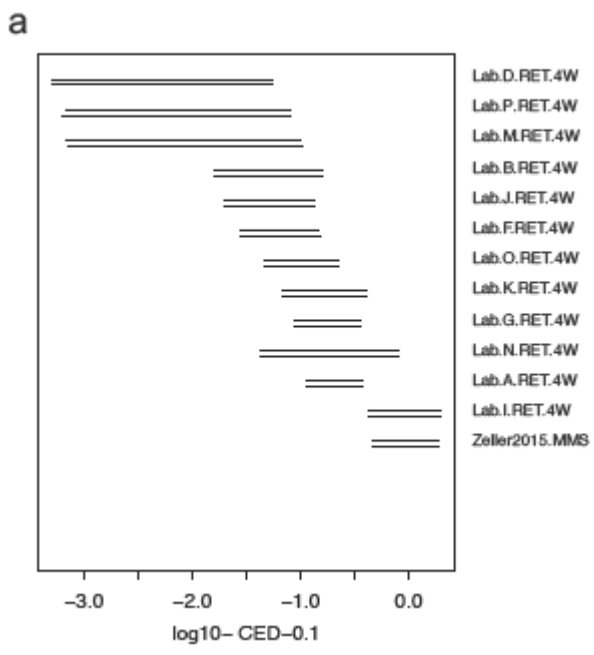


Figure 2: RET Week 4: *Pig-a* MF dose response data following exposure to ENU from the different laboratories was analysed using the BMD covariate approach, using BMRs of (a) 10% and (b) 100%. One MMS dose response data set from Zeller et al 2016 [17] was used to increase the precision of the BMD estimates [7]. The 4 parameter exponential (top horizontal line) and Hill (bottom horizontal line) 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. CED-0.1 is equivalent to, or another name for, BMD_{10}. X-axes are Log10.dose(mg/kg/day); Y-axes are laboratory.



b