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別添１

厚生労働科学研究費補助金

化学物質リスク研究事業

新規*in vivo*遺伝毒性試験である*Pig-a*遺伝子遺伝毒性試験の胎仔を含めた週齢および性差に関する開発研究（H25-化学-若手-008）

平成２６年度　総括研究報告書

研究代表者　堀端　克良

平成２７（２０１５）年　３月

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Ⅰ．総括研究報告

平成26年度　厚生労働科学研究費補助金（化学物質リスク研究事業）
総括研究報告書

新規 *in vivo* 遺伝毒性試験である *Pig-a* 遺伝子遺伝毒性試験の胎仔を含めた週齢および性差に関する開発研究（H25-化学-若手-008）

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研究要旨

幼児や妊婦（胎児）は、化学物質の遺伝毒性に対して脆弱であると考えられるが、それを定量的かつ簡便に評価する研究手法は未だに確立されていない。本研究課題では、近年開発された *Pig-a* 遺伝子遺伝毒性試験（以下、*Pig-a* アッセイ）について、性差および週齢差を踏まえた検討を行い、加えて、妊娠動物に遺伝毒性物質を投与した際の胎仔および新生仔における遺伝毒性影響を *Pig-a* アッセイにより評価することでその有用性を検証することを目的とする。

近年開発された *Pig-a* アッセイは米国および日本において産学官の共同研究が実施され、その成果を基に 2014 年に米国をリード国として OECD ガイドライン化に向けた SPSF が OECD に投稿された。本 SPSF には日本の協力が明示されている。本アッセイの OECD ガイドライン化達成に向け、本研究課題の推進により得られる研究成果は、日本国内の研究成果としてアピールすることができる。

A．研究目的

近年開発された *Pig-a* アッセイは、内在性遺伝子である *Pig-a* 遺伝子を標的としている。*Pig-a* 遺伝子はマウス、ラットそしてヒトなどのほとんどの哺乳動物で X 染色体上に座位しており、その遺伝子産物は GPI アンカー生合成の第一段階で機能する。*Pig-a* 遺伝子上に前進突然変異が生じると、GPI アンカーの生合成が阻害され、結果として細胞膜上に GPI アンカー結合タンパク質が提示されなくなる。この原理を利用し、*Pig-a* アッセイでは GPI アンカー結合タンパク質欠損赤血球の頻度をフローサイトメーターで実測値として検出し、*Pig-a* 遺伝子変異体頻度を求める。加えて、*Pig-a* アッセイでは遺伝毒性試験のためにトランスジェニック動物など特別な動物を使用する必要がなく、ヒトにも応用可能であり、解析にはマイクロリットル単位のごく微量の末梢血を使用し、また遺伝毒性の蓄積性を踏まえた解析が可能であると考えられている。その一方、*Pig-a* アッセイの有用性については、その特性から反復投与毒性試験への組み込みを勘案し、国内外において成熟ラットを用いた解析が主流であり、また、*Pig-a* アッセイは開発されてからの時間が浅いこともあり、その検出感度の性差、年齢差などは不明である。

研究代表者はこれまでに、幼若群は成熟群と比較し、高い *Pig-a* 遺伝子変異体頻度の上昇を示すことを明らかにしている。これらのことは、年齢差に応じた遺伝毒性の差を本アッセイにより評価できる可能性を示唆するものである。幼児や妊婦（胎児）は、化学物質の遺伝毒性に対して脆弱であると考えられるが、それを定量的かつ簡便に評価する研究手法は未だに確立されていない。本研究課題では、化学物質の子どもおよび胎児への遺伝毒性影響を検出可能な評価手法として *Pig-a* アッセイを提案し、その有用性を明らかにし、そして幼若動物や胎仔に与える遺伝毒性影響を明らかにすることを研究目的とする。本研究計画は三年間の研究計画とし、*Pig-a* アッセイの有用性について初年度は幼若マウスと成熟マウスでの雌雄差と週齢差の比較を行っ

た。今年度と最終年度はマウス胎仔を用いた解析を実施することを目標とした。

加えて、*Pig-a* アッセイの OECD ガイドライン化に向けた国内外での取り組みに対して、日本国内の研究成果をアピールするため、日本環境変異原学会の分科会である哺乳動物試験研究会（MMS 研究会：Mammalian Mutagenicity Study Group）に参画する機関中 17 機関が参加する共同研究を実施しており、研究代表者は総括世話人の役割を担っている。これらの日本国内の取り組みが評価され、平成 26 年末に米国をリード国として OECD に提出された SPSF（Standard Project Submission Form）に日本の貢献が明記された。それに伴い、上記共同研究を早期に達成する必要が生じたため、ラットを用いた *Pig-a* アッセイによる遺伝毒性評価を併せて実施する。

B． 研究方法

胎児マウス解析の予備的試験として、使用血液量を0.5μLまで抑制した条件での*Pig-a*アッセイを実施した。マウス*Pig-a*アッセイでは、末梢血を赤血球特異的蛍光抗体およびGPIアンカー結合タンパク質であるCD24特異的蛍光抗体により2重染色し、フローサイトメーターを用いて*Pig-a*変異体頻度を評価した。

MMS 研究会内でのラットを用いた共同研究では、日本国内独自の取り組みとして、全赤血球を標的とした RBC *Pig-a* アッセイに加えて、幼若赤血球を標的とした PIGRET 法を開発し、各機関内でエチルニトロソウレア（40 mg/kg および 10mg/kg 単回強制経口投与）を用いたバリデーション研究を実施し、線形回帰分析により各機関間の相関性を解析した。その後、各参加機関が分担し 24 種の化学物質の遺伝毒性評価を上記 RBC *Pig-a* アッセイおよび PIGRET 法により評価した。研究代表者は 24 種の化学物質中でアクリルアミドの遺伝毒性評価を担当した。経口投与後 7 日目での半数致死用量である 175mg/kg を最大容量とし、137.5 mg/kg、100 mg/kg、50 mg/kg および 25 mg/kg の用量で一群 6 匹 8 週齢雄の F344 ラットに強制経口投与し、投与前、投与後 1 週、2 週および 4 週目に尾静脈より採血し、RBC *Pig-a* アッセイおよび PIGRET 法により遺伝毒性を評価した。陽性対照として、エチルニトロソウレア（40 mg/kg）投与群も同時に設定した。

各投与群で得られた *Pig-a* 変異体頻度は、Steel の方法により、国立医薬品食品衛生研究所変異遺伝部の背景データ（溶媒投与群、N=95）と比較し、統計学的な解析を実施した。

（倫理面への配慮）

動物を用いた実験は、所属機関における「動物実験の適正な実施に関する規定」、わが国における「動物の保護及び管理に関する法律」、「実験動物の飼育及び保管等に関する基準」ならびに厚生労働省の所管する実施機関における動物実験等の実施に関する基本指針に準拠して行った。加えて、試験実施機関による動物実験に関する倫理委員会の承認を得るなど、実験動物に対する動物愛護を配慮の上で実施した。

C． 研究結果

1）極微量末梢血を用いた *Pig-a* アッセイ

胎児マウスから得られる末梢血量はごく微量であるため、本アッセイ系で解析可能な最少末梢血量を予備的試験として解析した結果、0.5μL でも十分解析可能であることが明らかになった。

2）ラットを用いた RBC *Pig-a* アッセイおよび PIGRET 法による遺伝毒性評価

研究代表者自らが RBC *Pig-a* アッセイおよび PIGRET 法の技術講習会を共同研究参加全機関に対して実施し、各参加機関それぞれの技術移管達成度を各機関において確認するバリデーション試験の線形回帰分析結果を図 1 に示す。両アッセイとも、全ての参加機関で強い正の相関が見られた。また、研究代表者が実施したアクリルアミドの遺伝毒性評価結果を図 2 に示す。アクリルアミドに関しては、両アッセイとも有意な差は検出されなかった。

D． 考察

1）極微量末梢血を用いた *Pig-a* アッセイ

本予備試験の結果から、胎児から得られる微量末梢血で *Pig-a* アッセイが実施可能であ

る予測を立てることができたため、次年度での解析への目処をつけることができた。

2）ラットを用いたRBC *Pig-a*アッセイおよびPIGRET法による遺伝毒性評価

バリデーション試験では各参加機関全てで正の相関が得られたため、両アッセイの技術移管は達成されたと判断される。今後、各機関において24種の化合物の遺伝毒性を分担して評価し、*Pig-a*アッセイのOECDガイドライン化達成に向け、米国に協力する形で日本国内の貢献を示す。

上記に関連したアクリルアミドの遺伝毒性評価について、RBC *Pig-a*アッセイおよびPIGRET法では陰性であったが、米国で先行して実施されている別手法の*Pig-a*アッセイの結果と同様の結果であり、RBC *Pig-a*アッセイおよびPIGRET法における再現性を示すことができたと考えられる。他方、トランスジェニック動物を用いた他のアクリルアミド遺伝毒性報告では陽性を示す場合があることが報告されている。*Pig-a*アッセイでは原理的に標的臓器が造血系のみであることから、造血系はアクリルアミドの遺伝毒性に関する標的臓器ではないと考えられる。

E．結論

これまでに得られたマウスを用いた研究成果によって、①遺伝毒性試験方法としての*Pig-a*アッセイとして見た場合、幼若動物を用いる方が感受性の高い試験を実施できる可能性が高いこと、②化学物質の遺伝毒性影響の視点から見た場合、成熟期よりも幼若期の方がより強い遺伝毒性影響を受ける可能性が高いこと、の２点が明らかになった。これにより、上記①については、*Pig-a*アッセイを実施する場合には使用動物の開始週齢をそれぞれの試験研究目的に応じて吟味した上で実施すべきであるということを提案するものであり、今後本アッセイを活用していく上で重要な情報となる。また、上記②については、幼若期における化学物質暴露に対する遺伝毒性リスクは成熟期よりも高いことを示唆するものであり、重要なリスク評価情報となる。

加えて、ラットを用いた日本国内の*Pig-a*アッセイ共同研究の推進により、本アッセイのOECDガイドライン化に向けた日本国内の貢献を強く示すことができる。

F．研究発表

1．論文発表

Gollapudi BB, Lynch AM, Heflich RH, Dertinger SD, Dobrovolsky VN, Froetschl R, Horibata K, Kenyon MO, Kimoto T, Lovell DP, Stankowski Jr LF, White PA, Witt KL, Tanir JY. The in vivo Pig-a assay: A report of the International Workshop On Genotoxicity Testing (IWGT) Workgroup. *Mutat Res.* in press.

Horibata K, Kono S, Ishigami C, Zhang X, Aizawa M, Kako Y, Ishii T, Kosaki R, Saijo M, Tanaka K. Constructive rescue of TFIIH instability by an alternative isoform of XPD derived from a mutated XPD allele in mild but not severe XP-D/CS. *J Hum Genet.* in press

Horibata K, Ukai A, Honma M. Evaluation of Rats' In Vivo Genotoxicity Induced by N-ethyl-N-nitrosourea in the RBC Pig-a, PIGRET, and gpt Assays. Genes and Environment. (2014) 36, 199-202.

Wakasugi M, Sasaki T, Matsumoto M, Nagaoka M, Inoue K, Inobe M, Horibata K, Tanaka K, Matsunaga T. Nucleotide Excision Repair-dependent DNA Double-strand Break Formation and ATM Signaling Activation in Mammalian Quiescent Cells. *J Biol Chem.* (2014) 289, 28730-28737.

Onami S, Cho YM, Toyoda T, Horibata K, Ishii Y, Umemura T, Honma M, Nohmi T, Nishikawa A, Ogawa K. Absence of in vivo genotoxicity of 3-monochloropropane-1,2-diol and associated fatty acid esters in a 4-week comprehensive toxicity study

using F344 gpt delta rats. *Mutagenesis.* (2014) 29, 295-302.

2．学会発表

堀端克良，鵜飼明子，本間正充，MMS/*Pig-a* 共同研究:アクリルアミドの遺伝毒性評価，日本環境変異原学会第43回大会，東京（2014.12）

山田雅巳，堀端克良，鵜飼明子，木本崇文，千藏さつき，伊東悟，武藤重治，宇野芳文，真田尚和，高島理恵，志賀野美幸，高沢博修，濵田修一，山本美佳，堀妃佐子，堤絵梨，和田邦生，前田晃央，小坂瑞樹，木村葵，菊月隆太，荻原庸介，京谷恭弘，足立秀樹，上松泰明，吉田唯真，成見香瑞範，福田隆之，鈴木裕太，後藤玄，森田健，本間正充，*Pig-a*/PIGRET アッセイに関する短期試験への有用性：MMS 共同研究報告，日本環境変異原学会第43回大会，東京（2014.12）

堀端克良，共同研究報告 I:*Pig-a* assay 進捗状況報告，哺乳動物試験研究会第65回定例会，東京（2014.12）

堀端克良，鵜飼明子，石川恵生，菅野絢子，木本崇文，本間正充，マウス、ラットおよびヒト由来のごく微量末梢血を用いて解析可能な *Pig-a/ PIG-A in vivo* 突然変異試験法，第37回日本分子生物学会年会，横浜（2014.11）

Horibata K, Honma M. ESTABLISHMENT OF HUMAN *PIG-A* ASSAY AND APPLICATION TO GENOTOXICITY MONITORING OF CANCER CHEMOTHERAPEUTIC PATIENTS, 第73回日本癌学会学術総会，横浜（2014.9）

堀端克良，共同研究報告 I:*Pig-a* assay 進捗状況報告，哺乳動物試験研究会第64回定例会，熱海（2014.6）

G．　知的財産権の出願・登録状況

1．特許取得

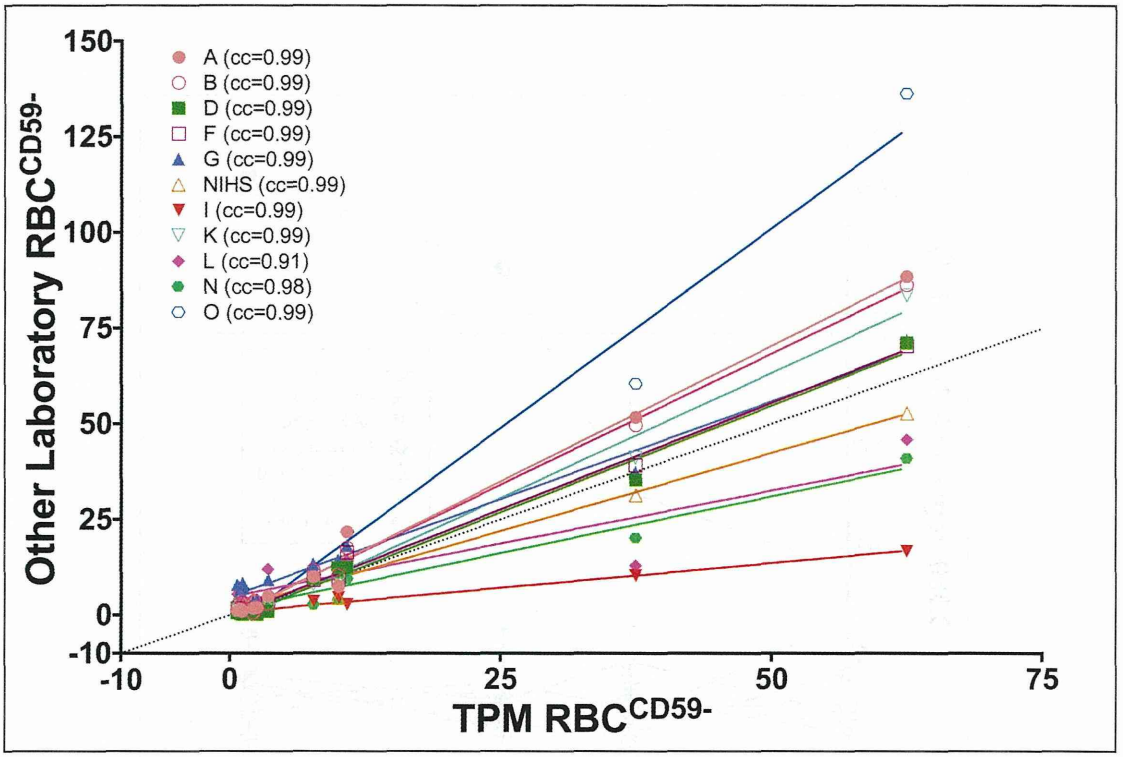
なし

2．実用新案登録

なし

3．その他

なし



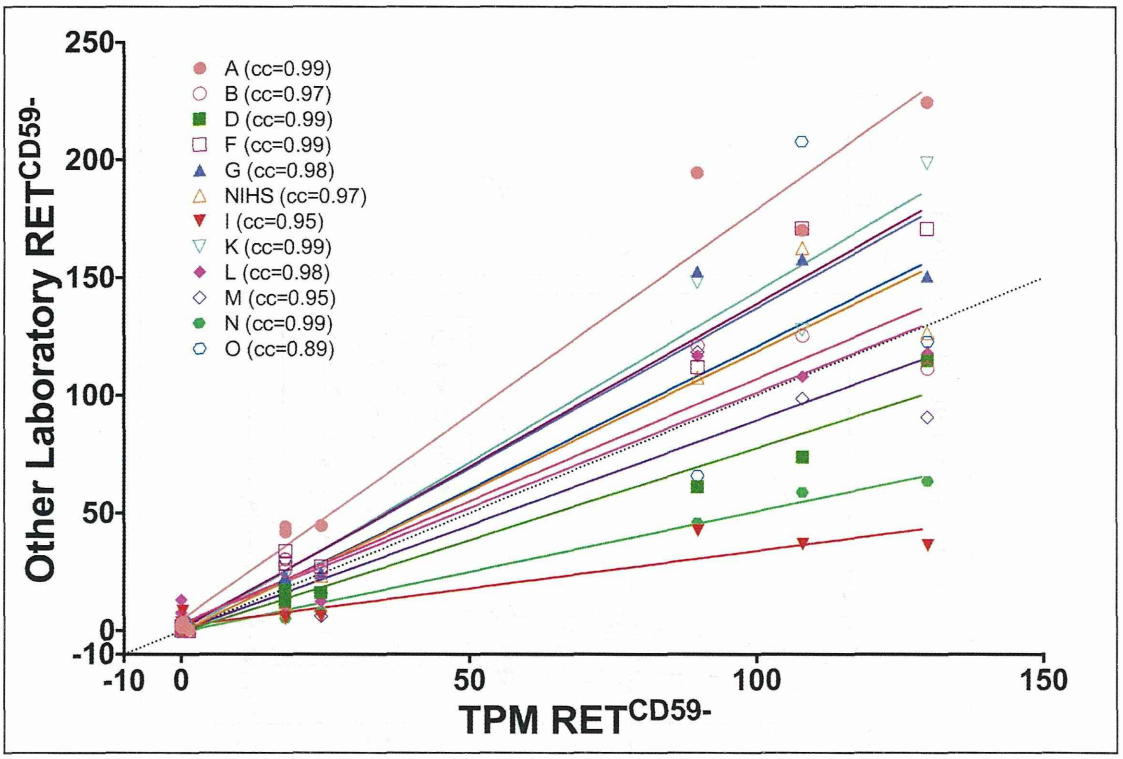
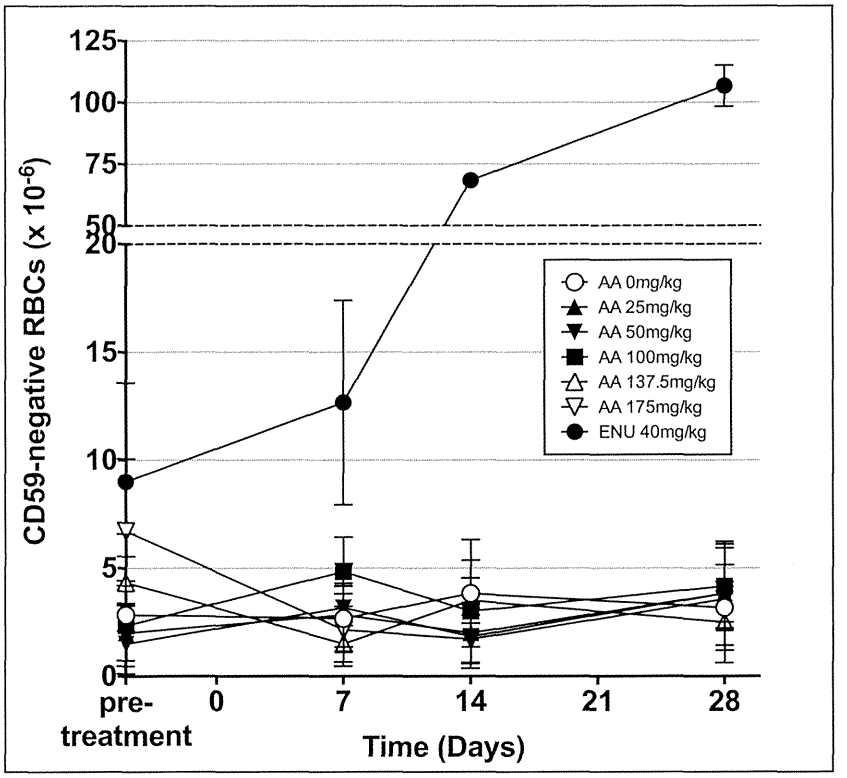


図１．線形回帰分析と相関係数（上，RBC *Pig-a*アッセイ．下，PIGRET法）



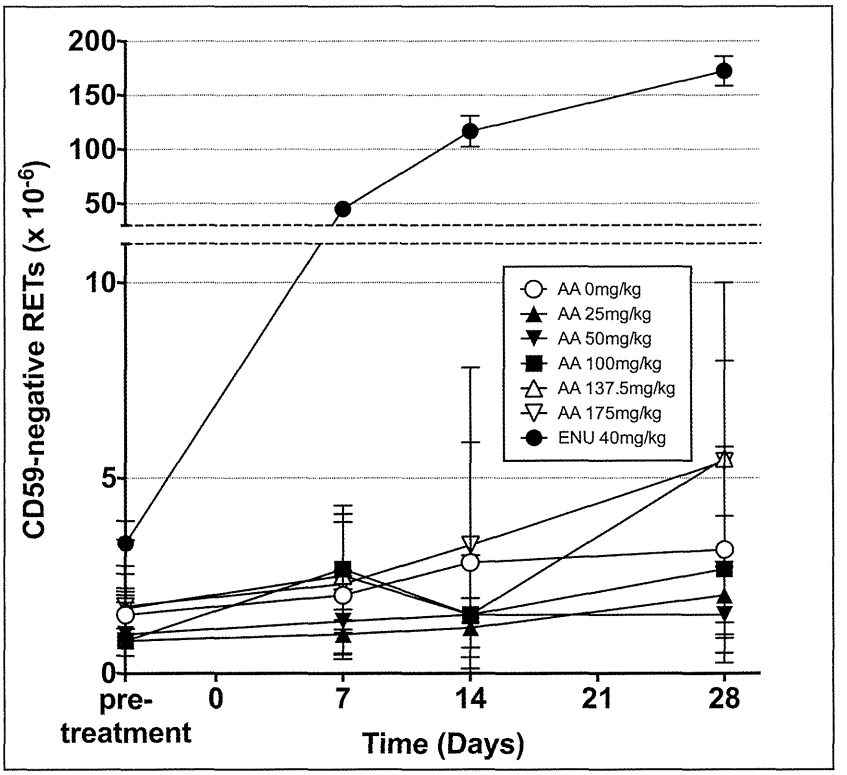


図２．ラットを用いたアクリルアミドの遺伝毒性評価（上，RBC *Pig-a*アッセイ．下，PIGRET法）

別添５

II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
該当無し							

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Gollapudi BB, Lynch AM, Heflich RH, Dertinger SD, Dobrovolsky VN, Froetschl R, Horibata K, Kenyon MO, Kimoto T, Lovell DP, Stankowski Jr LF, White PA, Witt KL, Tanir JY	The in vivo Pig-a assay: A report of the International Workshop On Genotoxicity Testing (IWGT) Workgroup.	*Mutation Research*	in press	in press	2015
Horibata K, Kono S, Ishigami C, Zhang X, Aizawa M, Kako Y, Ishii T, Kosaki R, Saijo M, Tanaka K.	Constructive rescue of TFIIH instability by an alternative isoform of XPD derived from a mutated XPD allele in mild but not severe XP-D/CS.	*Journal of Human Genetics*	in press	in press	2015
Horibata K, Ukai A, Honma M	Evaluation of Rats' In Vivo Genotoxicity Induced by N-ethyl-N-nitrosourea in the RBC Pig-a, PIGRET, and gpt Assays.	*Genes and Environment*	36	199-202	2014

Wakasugi M, Sasaki T, Matsumoto M, Nagaoka M, Inoue K, Inobe M, Horibata K, Tanaka K, Matsunaga T.	Nucleotide Excision Repair-dependent DNA Double-strand Break Formation and ATM Signaling Activation in Mammalian Quiescent Cells.	*The Journal of Biological Chemistry*	289	28730-7	2014
Onami S, Cho YM, Toyoda T, Horibata K, Ishii Y, Umemura T, Honma M, Nohmi T, Nishikawa A, Ogawa K.	Absence of in vivo genotoxicity of 3-monochloropropane-1, 2-diol and associated fatty acid esters in a 4-week comprehensive toxicity study using F344 gpt delta rats.	*Mutagenesis*	29	295-302	2014

III. 研究成果の刊行物・別刷

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Community address: www.elsevier.com/locate/mutres

The *in vivo Pig-a* assay: A report of the International Workshop On Genotoxicity Testing (IWGT) Workgroup☆

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[d] *Litron Laboratories, Rochester, NY, USA*
[e] *Federal Institute for Drugs and Medical Devices, Bonn, Germany*
[f] *National Institute of Health Sciences, Tokyo, Japan*
[g] *Pfizer Global Research and Development, Groton, CT, USA*
[h] *Teijin Pharma, Tokyo, Japan*
[i] *St. George's, University of London, UK*
[j] *BioReliance, Rockville, MD, USA*
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[l] *National Institutes of Health, National Institute of Environmental Health Sciences, Division of the National Toxicology Program, Research Triangle Park, NC, USA*
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Mutation

ABSTRACT

The *in vivo Pig-a* assay uses flow cytometry to measure phenotypic variants for antibody binding to cell surface glycosylphosphatidylinositol (GPI)-anchored proteins. There is good evidence suggesting that the absence of antibody binding is the result of a mutation in the endogenous X-linked *Pig-a* gene, which forms the rationale for the assay. Although the assay has been performed with several types of hematopoietic cells and in a variety of mammalian species, including humans, currently it is optimized only for measuring CD59-deficient (presumed *Pig-a* mutant) erythrocytes in the peripheral blood of rats. An expert workgroup formed by the International Workshop on Genotoxicity Testing considered the state of assay development and the potential of the assay for regulatory use. Consensus was reached on what is known about the *Pig-a* assay and how it should be conducted, and recommendations were made on additional data and refinements that would help to further enhance the assay for use in hazard identification and risk assessment.

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1. Introduction

1.1. Background to this report

The *in vivo Pig-a* assay was first described for rats and mice in 2008 [1–4], and received almost immediate interest as a potential assay for evaluating the *in vivo* mutagenicity of new and existing substances. Subsequently, formal and informal workshops and presentations on the assay were held at meetings of the US Environmental Mutagenesis and Genomics Society and the Health and Environmental Sciences Institute (HESI) of the International Life Sciences Institute [5]. A Workgroup, made up of experts from academic, regulatory, and industrial laboratories, was formed in 2012 under the auspices of the International Workshop on Genotoxicity Testing (IWGT) to review the development of the *Pig-a* assay in the context of safety assessment strategies. The remit of the Workgroup was to consider the underlying science of the *Pig-a* assay and technical considerations for the assay protocol with a view to assay acceptance in a regulatory context and to recommend where and how further progress on developing the assay can be made. Subsequently, two meetings of the Workgroup were held in 2013, one in conjunction with the HESI Genetic Toxicology Technical Committee annual meeting in Washington, DC, on April 15 and the second at the IWGT meeting in Foz do Iguaçu, Brazil, on October 31–November 1. This report describes consensus statements on the *Pig-a* assay developed by the IWGT Workgroup.

1.2. Principle of the assay

The *Pig-a* assay is based on the identification of mutant cells that have an altered repertoire of cell surface markers (Fig. 1). The assay was developed from an understanding of the molecular nature of a rare human acquired genetic disorder, paroxysmal nocturnal hemoglobinuria (PNH) (reviewed [6–8]). The *Pig-a* gene (phosphatidylinositol glycan, class A gene) codes for the catalytic subunit of an *N*-acetyl glucosamine transferase that is involved in an early step of glycosylphosphatidylinositol (GPI) biosynthesis. GPI anchors an assortment of protein markers (*e.g.*, CD59, CD24, CD55) to the exterior surface of the cytoplasmic membranes of higher eukaryotes. In mammals, *Pig-a* (nomenclature: *PIG-A*

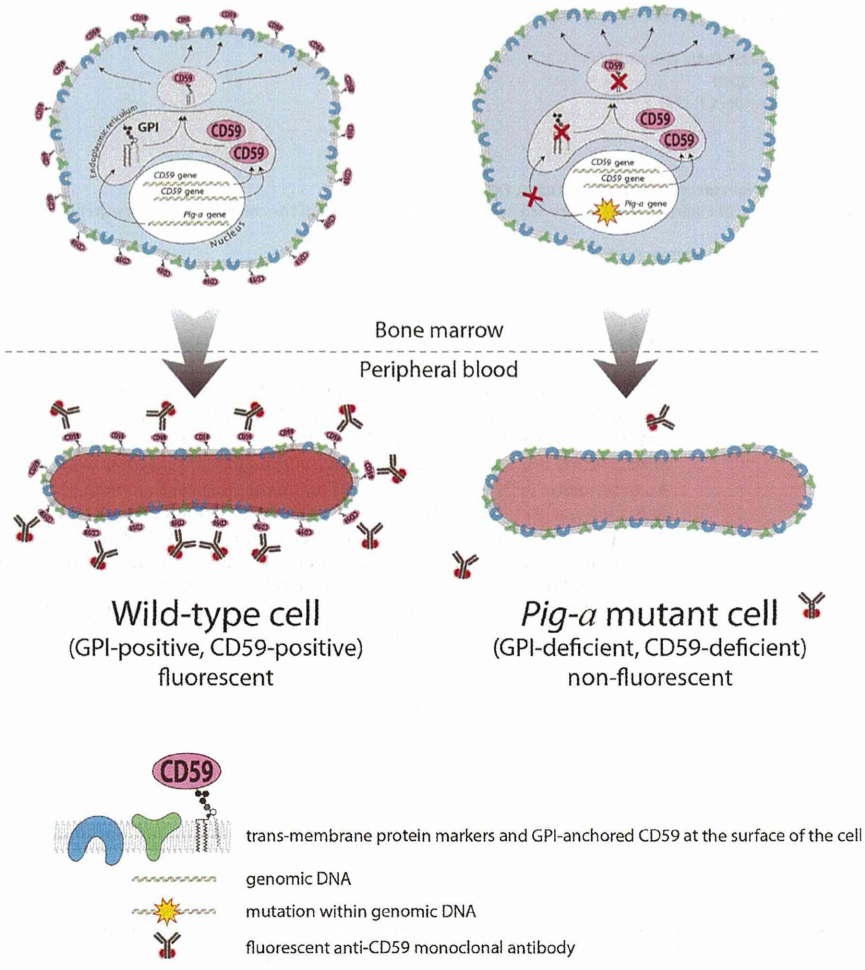

Fig. 1. Principle of the rat erythrocyte *Pig-a* assay. In wild-type nucleated erythroid precursor cells (upper left), glycosylphosphatidylinositol (GPI) anchors are synthesized in a series of steps at the endoplasmic reticulum (ER). The first step is catalyzed by the product of the X-linked *Pig-a* gene; all other steps of the GPI synthesis pathway are catalyzed by enzymes encoded by autosomal genes. The GPI core structure consists of 3 mannoses, 1 glucosamine, and 1 phosphatidylinositol, with 2 or 3 hydrophobic tails imbedded in the ER lipid bilayer membrane (a more detailed description of GPI structure and synthesis can be found in [7]). In the final stage of biosynthesis, a surface marker (*e.g.*, CD59 protein, which is synthesized independently) is attached to the GPI anchor and the assembly is transported to the surface of the cell. *Pig-a* mutant cells (upper right) are deficient in GPI anchor synthesis, and thus GPI-anchored markers are not transported to the cytoplasmic membrane. The *Pig-a* assay is conducted with peripheral blood erythrocytes having no nuclei (lower, left and right). At the surface of the cytoplasmic membrane, GPI anchored CD59 reacts with fluorescent anti-CD59 antibodies which make the whole wild-type cell fluoresce (lower left). *Pig-a* mutant cells do not express CD59 on their surface and thus are not fluorescently labeled (lower right). Flow cytometry is used to quantify the number of wild-type and mutant cells and estimate a *Pig-a* mutant frequency.

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in humans and other primates; *Pig-a* in mice and rats) is an X-linked gene present as a single functional copy in cells from both males and females. Other genes involved in GPI biosynthesis are autosomal and have two functional copies. A single inactivating mutation in the *Pig-a* gene is sufficient to make a cell deficient in GPI anchors and, as a consequence, deficient in surface-bound GPI-anchored markers. Since it is exceedingly unlikely that anchor deficiency would occur due to inactivating mutations in both copies of the autosomal genes involved in GPI synthesis, measuring GPI deficiency is considered 'virtually equivalent' to measuring *Pig-a* mutation.

The assay distinguishes the *Pig-a* mutant phenotype from the wild-type phenotype by labeling cells with fluorescent antibodies against a GPI-anchored marker or markers [8] (Fig. 1). Wild-type cells bind marker-specific antibodies and fluoresce, while mutant cells do not bind the antibodies and do not fluoresce. Cells are analyzed using high throughput flow cytometry to quantify wild-type (*i.e.* labeled) and rare mutant (*i.e.* unlabeled) cells. There are multiple commercially available antibodies, stains, and reagents suitable for designing *Pig-a* assays in different cell types and in different mammalian species.

The use of flow cytometry for detecting cells deficient in surface markers imposes certain constraints on the tissues amenable for the *Pig-a* assay. For instance, the samples must be prepared as single-cell suspensions, and the cells should not be subjected to treatments that may alter the cell surface membrane (*e.g.*, proteolytic digestion). Currently, these two requirements have limited the *in vivo* application of the *Pig-a* assay to hematopoietic tissue (peripheral blood erythrocytes and white blood cells, and, to a lesser degree, bone marrow).

A combination of theory and experimental evidence indicates that *Pig-a* mutations occurring in bone marrow cells result in the emergence of the mutant cells that are measured in peripheral blood [9,10]. The appearance of *Pig-a* mutant cells in peripheral blood exhibits a time delay that is dependent upon the cell and GPI-anchor turnover rates, as well as the trafficking time from the bone marrow to the periphery for the specific cell type. Red blood cells (RBCs) are the most practical cell type for performing the assay since microliter volumes of blood contain sufficient quantities of RBCs for the enumeration of the rare mutants required for a successful *Pig-a* assay. The abundance of RBCs in peripheral blood allows serial blood collections from even small laboratory animals and thus permits longitudinal studies to be conducted on the same set of animals. In addition, the small fraction of newly formed erythrocytes, *i.e.*, reticulocytes (RETs), can be distinguished by appropriate staining. RETs have a rapid turnover rate (few days), and express the mutant phenotype originating in the bone marrow faster than the population of total RBCs in peripheral blood. Finally, at least for the rat assay, *Pig-a* mutant erythrocytes act as though they have a neutral phenotype [9], meaning that mutant cells are not subjected to negative selection and can accumulate with repeated dosing, potentially increasing the sensitivity of the assay. Observations concerning *Pig-a* mutant manifestation in the peripheral blood and mutant accumulation with repeat doses are consistent with what occurs in other *in vivo* gene mutation assays [11,12], and lend support to the mutational origin of the phenotype measured in the *Pig-a* assay.

1.3. Potential for translation of the endpoint from experimental models to humans

The pathway for GPI biosynthesis is conserved in most mammalian species, including common laboratory animals, such as mice, rats and monkeys, and in humans. In fact, approaches for scoring *Pig-a/PIG-A* mutant cells have been described for all these species [1–3,13–16]. Although credit for the original flow cytometric methodology for measuring mutant cells goes to David Araten and Lucio Luzzatto, who described *PIG-A* assays for human granulocytes and RBCs [17], human and monkey assays are now much less well developed than the rat assay. Also, while comparable methodology to the rat assay is available for the mouse, at present there is considerably less experience conducting the *Pig-a* assay in mice.

The ability to evaluate *Pig-a* mutants in both humans and laboratory animals means that hypotheses about the responsiveness of humans to potential mutagen exposures can be tested in animal models. Also, the human *PIG-A* assay may have value in clinical settings, where *Pig-a* assays conducted in laboratory animals could provide a translational biomarker for endogenous mutation *in vivo*. For example, the *Pig-a* and *PIG-A* assays could be used for monitoring the long-term effects of genotoxic chemotherapy [15] and aid in estimating the likelihood of developing secondary malignancies. Additionally, the *PIG-A* assay could be used in epidemiology studies for monitoring the health status (mutation accumulation) in populations exposed to potentially adverse environments, including occupational or accidental exposures to hazardous chemicals.

2. Topics discussed by the Workgroup

2.1. Placement of the assay within genotoxic testing strategies

The *Pig-a* assay should be considered an appropriate *in vivo* follow-up to positive results in bacterial and *in vitro* mammalian cell gene mutation assays. However, unless bone marrow exposure to the parent compound or to its metabolite(s) can be demonstrated directly or indirectly *via* plasma or by a reduction in the percentage of RETs, caution must be exercised in interpreting negative results. Moreover, based on analysis of the testing performed to date (see Section 2.2.3), the assay is not limited by any requirement of the test agent for metabolic activation. Although *Pig-a* is an endogenous gene, it is located on the X chromosome, and there is only one functional X chromosome. Thus, the Workgroup recognizes that the assay may not be suitable as a follow-up to either *in vitro* clastogenicity or aneugenicity findings since such events could potentially lead to cell death rather than mutation [18]. However, there currently is limited experience on the ability of the assay to detect clastogens or aneugens and the Workgroup considered the available information insufficient to justify a data-driven recommendation on this issue. Results from the *Pig-a* assay also could be used to build weight of evidence for the *in vivo* mutagenicity of a test agent, as might occur in cancer mode-of-action evaluations. Finally, the Workgroup noted the potential of using the *Pig-a* assay, as a measure of gene mutation, to complement the micronucleus (MN) assay, which measures clastogenicity/aneugenicity. Both can be readily included in routine *in vivo* safety evaluations, especially when the assays are integrated into subchronic (28 or 90 day) treatment protocols.

2.2. State of validation

Multi-laboratory trials initiated by Litron Laboratories [19], the Japanese Research Group [20], and the HESI initiative [5] have contributed to establishing protocols for the assay, testing the inter-laboratory reproducibility of the assay, and in expanding the number of agents tested.

2.2.1. Species, strain, and cell type

The assay has been investigated extensively only in rats. While the strain of rat may influence the absorption, distribution, metabolism, and elimination (ADME) of a test material and thus

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affect the assay outcome, this is not anticipated to influence the biology of the endpoint; however, strain variation, if any, deserves further investigation.

Mammalian species used in other toxicological/investigational studies are amenable for use, theoretically, and there are a number of publications on *Pig-a* (or *PIG-A*) assays using other species (*e.g.*, human [15,17], mouse [2,13]). However, a recommendation for their routine use in safety assessment studies cannot be made at this time because standard protocols have not yet been evaluated extensively or published.

The assay has been extensively investigated using RBCs and RETs. Measuring the *Pig-a* mutant phenotype in RETs has the advantage of observing an induced response more quickly than in RBCs and has the theoretical advantage of mitigating any effects of immune lysis (which does not seem to be a major factor in rats [9]). For routine screening, however, it is recommended that RBCs also should be investigated for GPI-anchored protein deficiency because of the ability to score a much larger sample size of cells. Additional studies have used other cell types in the *Pig-a* assay (*e.g.*, lymphocytes [3,4], bone marrow erythroid cells [10]); but while these cell types are potentially useful, a recommendation for their routine use in safety assessment studies cannot be made at this time.

2.2.2. Intra/inter-laboratory reproducibility

The Workgroup could not identify any studies specifically intended to test the intra-laboratory reproducibility of the assay beyond assaying technical replicates (*e.g.*, [21]; also see results in Fig. 2 and Table 3). There is, however, good evidence for a high degree of inter-laboratory transferability and reproducibility based on the results with several potent and weak mutagens (and low doses of potent mutagens) that were tested in a systematic manner as part of the Litron trial [22–27]. This trial employed common protocols (refined for each stage of the trial), and common reagents, although the rat strains and flow cytometers used were at the discretion of the participating laboratory. Stage I consisted of information gathering, and Stage II tested the transferability of an anti-CD59 antibody-based method using data from Litron Laboratories as the comparator. Stage II was based on assays employing acutely administered doses of *N*-ethyl-*N*-nitrosourea (ENU), and involved 14 laboratories [22]. The results demonstrated a high level of agreement, and provided confidence in extending the studies to investigate the performance characteristics of the assay further. Stage III utilized a 28-day repeat-dose design, and experiments were performed with ENU [23], dimethylbenz[*a*]anthracene (DMBA; [24]), *N*-methyl-*N*-nitrosourea [25], benzo[*a*]pyrene (BaP; [26]), and 4-nitroquinoline-1-oxide (4-NQO; [27]) in at least 2 laboratories per agent. Results from these studies also showed a high degree of concordance among laboratories. In addition, these data provided the impetus for establishing a method to dramatically increase the number of cells evaluated per sample through the use of immunomagnetic separation technology [21]. Stage IV studies have been conducted with these updated methods and demonstrate improved statistical power [21,28,29], but only a fraction of the completed work has been published or available to the Workgroup in a form that could be used to assess intra- and inter-laboratory reproducibility.

Results from the Japanese trial, using a different staining protocol, have begun to appear, and demonstrate a similar degree of inter-laboratory reproducibility [20]. Therefore, the Workgroup concluded that the available data show that the assay is both robust and demonstrably reproducible within and across laboratories for test agents with a range of mutagenic potency. The primary variable influencing the assay outcome across laboratories appears to be sample labeling and processing for flow cytometry.

Regarding assessments of sample labeling and processing, the Workgroup acknowledged that performing independent studies with the same test agent is not always the most effective way of evaluating the impact of these factors on mutant frequency measurements. Rather, results from blood samples processed at one site on multiple occasions, and blood samples split among multiple sites, can often be more informative. For instance, Fig. 2 shows the results from 15 Sprague Dawley rats treated with either the vehicle (*i.e.*, negative control) or 1 of 2 dose levels of thiotepa. Four weeks after cessation of treatment for 28-days, blood samples were collected and processed by Litron personnel (at Rochester, NY) for same-day determination of mutant RET and RBC frequencies. A second aliquot of each whole blood sample was maintained in a refrigerator for next-day labeling and analysis at Litron, while a third set of coded aliquots was shipped overnight to Groton, CT to collaborators at Pfizer for labeling and flow cytometric analysis. All "next day" blood samples were maintained as whole blood in EDTA-coated vials until processing occurred. For the overnight shipment, the samples were kept cold, but not frozen. Both next-day analyses produced *Pig-a* mutant frequencies that were similar to those of the assays conducted on fresh samples (Fig. 2). Another study, using a different labeling protocol, demonstrated that the shipment of refrigerated blood samples had little effect on the measurement of *Pig-a* mutant frequencies [20]. These results strongly support the Workgroup's conclusion that, where there are sufficiently trained personnel, the sample labeling and flow cytometric analysis procedures are highly reproducible within and across laboratories.

2.2.3. Test agent coverage

A list of 41 agents tested in the rat *Pig-a* assay was compiled by the Workgroup (Table 1). Most of the agents identified by the Workgroup were genotoxic in one or more tests, including 26 that were Ames' test positive. The testing that has been conducted identified most of the agents expected to be positive as positive in the assay. Besides direct-acting simple alkylating agents, positive responses were detected from a number of chemicals that require metabolic activation in order to manifest their genotoxicity, including 2-acetylaminofluorene, aflatoxin B1, aristolochic acids, BaP, cyclophosphamide, diethylnitrosamine (DEN), dibenzo[*a*,*l*]pyrene, DMBA, and urethane. It is apparent from these observations that sufficient reactive metabolites from these agents damage the DNA of erythroid precursor cells to produce a response in the assay, even when other tissues may be responsible for the metabolism of these compounds. None of the unanticipated negative responses in the assay were clearly associated with a requirement for metabolic activation. Of note, the magnitude of positive responses indicates that the dynamic range of the *Pig-a* assay is at least 2–3 orders of magnitude: while background frequencies were $\leq 5 \times 10^{-6}$ (see Section 2.5.1), induced frequencies for the most potent mutagen tested to date, ENU, were (depending on the dose) in excess of 1000×10^{-6} (*e.g.*, [9]).

The Workgroup found a few examples of responses produced by genotoxic agents that illustrated both the strengths and the limitations of the assay. Urethane, a genotoxicant requiring metabolic activation not present in many *in vitro* test systems [30], was positive in the assay. DEN, which is considered to be a liver-specific genotoxicant that is often negative for assays of bone marrow genotoxicity (like the erythrocyte MN assay), initially tested negative in the assay using the 'basic' protocol [24]. Subsequent testing using an immunomagnetic separation protocol produced a positive response [31]. Etoposide, azidothymidine (AZT), and 5-fluorouracil tested negative in the assay but all have mutagenic mechanisms which would be anticipated to be difficult to detect with an X-linked reporter gene, and only the basic protocol and/or acute dosing have been used for testing to date. Finally, aflatoxin B1 was positive in the assay, but only following subchronic, and not acute dosing,

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Table 1
Agents tested in the rat *Pig-a* assay.

Compound	CAS no.	Chemical class	Expected outcome[a]	Rat *Pig-a* result		References	Comments
				Acute exposure[b]	Subchronic exposure[c]		
2-Acetylaminofluorene	53-96-3	Aromatic amine	+	+	+	[33]; Novartis (unpublished)	Requires metabolic activation; 2 labs
Acetaminophen	103-90-2	Hydroxyaniline	–	–HT	–HT	Jannsen (unpublished)	Potent liver toxicant
Acrylamide	79-06-1	α,β-unsaturated amide	–/Weak+		–/? HT	FDA-NCTR (unpublished)	TGR, *Hprt* lymphocyte positive (2 mo treatment)
Aflatoxin B1	1162-65-8	Mycotoxin	+	–HT	Weak+	Jannsen (unpublished)	Requires metabolic activation by CYP3A4 (not highly expressed in rats compared to humans)
o-Anthranilic acid	118-92-3	Aromatic amine	–	–HT	–HT	[33]	Non-alerting structure
Aristolochic acids	313-67-7	Aromatic nitro	+	+	+	[32]; Novartis (unpublished)	2 labs; weak *in vivo* MN
Azathioprine	446-86-6	Aromatic nitro	–/Weak +	Weak+	Weak+	[33]	Immunotoxicant
Azidothymidine	30516-87-1	Nucleoside analog	–/Weak+	–Basic (7 days)		[60]	*In vivo* MN positive; *Tk* mutation positive (mouse)
Benzo[*a*]pyrene	50-32-8	PAH	+	+	+	[26,29,37,52]	Requires metabolic activation; 3 labs
2-Butoxyethanol	111-76-2	Ethylene glycol derivative	–	–HT	–HT	Pfizer (unpublished)	Hemolytic agent
Chlorambucil	305-03-3	Alkylator; nitrogen mustard	+	+	+	[33,53]; Novartis (unpublished)	2 labs; twice at one lab
4-Chloro-1,2-diaminobenzene	95-83-0	Aromatic amine	–(Male) +(Female)	–HT (male)	–HT (male)	GSK (unpublished)	*In vivo* MN positive acute exposure only; Females not tested
Cisplatin	15663-27-1	Antineoplastic; crosslinker	+	+	+	[54,58]	2 labs
Cyclophosphamide	6055-19-2	Alkylator, nitrogen mustard	Weak+	+	+	[33,56,58]	Requires metabolic activation; strong *in vivo* MN positive; 3 labs
Dibenzo[*a,l*]pyrene	191-30-0	PAH	+	+		Roche (unpublished)	Requires metabolic activation
Diethylnitrosamine	55-18-5	Nitrosamine	–(Blood)		–Basic; weak + HT	[24,31]	Requires metabolic activation; weak *in vivo* MN
[1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, (EDAC)	25952-53-8	Carbodiimide	–		–HT	[61]	Ames and *in vitro* MN positive; Tested up to MTD, rapid degradation into non-mutagenic EDAU in acid conditions and in aqueous solutions (SD rats)
7,12-Dimethyl-benz[*a*]anthracene	57-97-6	PAH	+	+	+	[1,20,24,37,52]; Janssen (unpublished)	Requires metabolic activation; 5 labs; twice at one lab (SD & Wistar rats)
1,2-Dimethylhydrazine hydrochloride	306-37-6	Hydrazine	–(Blood)	–Basic		Pfizer (unpublished)	
Ethylmethane sulfonate	62-50-0	Alkylator	+	+	+	[56,62]; FDA-NCTR (unpublished)	3 labs
N-Ethyl-*N*-nitrosourea	759-73-9	Alkylator	+	+	+	[1,3,9,20,22,23,37,51,56,57,62]; PBR Laboratories (unpublished)	Multiple labs (13+ for 3 day; 5 for 28-day)

Table 1 (*Continued*)

Compound	CAS no.	Chemical class	Expected outcome[a]	Rat *Pig-a* result		References	Comments
				Acute exposure[b]	Subchronic exposure[c]		
Etoposide	33419-42-0	Topoisomerase inhibitor	–	–Basic	–Basic	Pfizer (unpublished)	Bacterial specific mutagen
5-Fluorouracil	51-21-8	Pyrimidine analog	–/?	–HT		NCDSER (unpublished)	Toxicity to red blood cells observed; positive in concurrent *in vivo* MN test
Furan	110-00-9	Heterocycle	–		–Basic (56 days)	[63]	Requires metabolic activation by CYP2E1; concurrent spleen lymphocyte *Hprt* and *Pig-a* and *cII* liver mutation assays were negative
Hydroxyurea	127-07-1	Inhibits DNA replication	–	–HT	–HT	[33]	
Ionizing radiation	N/A		+	+(1 and 4 days)		[58]; Litron Laboratories (unpublished)	2 labs
Isopropylmethane sulfonate	926-06-7	Alkylator	+	+(1 and 3 days)	+	Pfizer (in preparation)	
Isopropyltoluene sulfonate	2307-69-9	Alkylator	+	+(1 day)		Pfizer (unpublished)	
Melamine	108-78-1	Triaminotriazine	–	–HT		NCDSER (in preparation)	
Melphalan	148-82-3	Alkylator; nitrogen mustard	+	+	+	[33,53]; Novartis (unpublished)	2 labs; twice at one lab
Methylmethane sulfonate	66-27-3	Alkylator	Weak+	+	+	[33]; Roche (unpublished)	2 labs
Methylphenidate	113-45-1	Phenylethylamine derivative (psycho-stimulant)	–		–Basic (21 days)	[55]	
4-Nitroquinoline-1-oxide	56-57-5	Aromatic *N*-oxide	+	+	+	[20,27,37,52,56,64]; AbbVie (unpublished)	4 labs; twice at one lab
N-Nitroso-N-methylurea	684-93-5	Alkylator	+	+	+	[25,37,52,65]	3 labs; twice at one lab (basic and HT)
1,3-Propane sultone	1120-71-4	Alkylator	+	+	+	[28,53]; Novartis (unpublished)	2 labs; twice at one lab
Pyrene	129-00-0	PAH	–	–HT	–HT	[29]	
Sodium chloride	7647-14-5	Salt	–	–HT	–HT	[33]	
Sulfisoxazole	127-69-5	Aryl sulfonamide	–	– HT	–HT	[33]	
Temozolomide	85662-93-1	Imidazotetrazine; (Alkylating agent)	+	+(5 days)		Roche (unpublished)	
Thiotepa	52-24-4	Aziridine	+	+	+	[33,53]; Novartis (unpublished); Litron/Pfizer (summarized within)	2 labs; twice at one lab and a shipment study with a third lab
Urethane	51-79-6	Carbamate	+		+	[66]	Requires metabolism that is not available *in vitro*; TGR positive; weak effects

[a] Based on expert judgment of Ames and *in vitro* mammalian gene mutation data, mechanism of genotoxicity, and tissue distribution *in vivo*.
[b] Acute is 3 consecutive days dosing unless otherwise noted.
[c] Subchronic is 28 consecutive days dosing unless otherwise noted.
HT, immunomagnetic separation; only noted when a compound is negative; basic, no immunomagnetic separation; only noted when a compound is negative; TGR, transgenic rodent; –, negative; +, positive;?, equivocal; N/A, not applicable; ND, no data; PAH, polyaromatic hydrocarbon; NCDSER, National Shanghai Center for Drug Safety Evaluation and Research.

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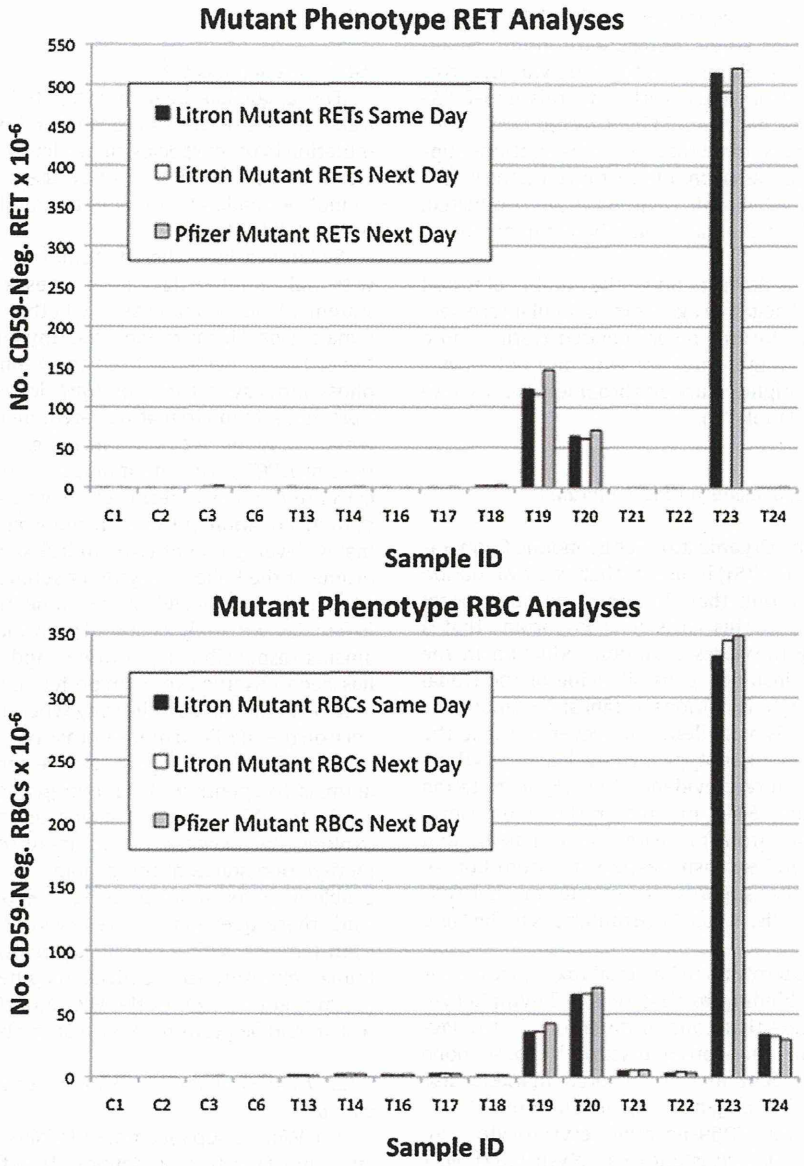

Fig. 2. *Pig-a* data from 15 individual male Sprague Dawley rats treated with vehicle (C) or 1 of 2 dose levels of thiotepa (T). All animals were treated daily for 28 consecutive days. 'C' animals were treated with the vehicle, T13-T18 animals were given 0.492 mg thiotepa/kg/day, while animals T19–T24 were given 10 mg thiotepa/kg/day for 17 days and 5 mg/kg/day for the remaining 11 days. Blood samples were collected 28 days after the last treatment and processed by Litron Laboratories' personnel for same-day determination of *Pig-a* mutant RET and RBC frequencies. A second aliquot of each whole blood sample was maintained in a refrigerator overnight prior to labeling and analysis at Litron (Rochester, NY), while a third set of aliquots was shipped to collaborators at Pfizer (Groton, CT) for labeling and flow cytometric analysis. All "next day" blood samples were maintained as whole blood in EDTA-coated vials until processing occurred. In the case of the overnight shipment, vials were kept cold, not frozen. Each bar represents the *Pig-a* mutant frequency for each individual rat's blood sample at one of the 3 conditions tested. Note that some bars for the control and low-dose-treated rats are hardly visible on the figure. The results, which show extreme rat-to-rat variability among the high-dose animals at this late blood collection time point, indicate that across a wide dynamic range, *Pig-a* mutant frequencies are highly reproducible within and between laboratories.

while acrylamide was at best inconclusive after subchronic testing; acrylamide was not tested using acute treatment.

Based on these observations, the Workgroup concluded that definitive negative calls should be based on data from the most sensitive protocols (*i.e.*, using immunomagnetic separation), and tests conducted to the maximum tolerated dose (MTD) or the limit dose (see Section 2.4.2). This recommendation extends to agents anticipated to be negative, including Ames' negative agents. Of the 8 agents tested that generally are considered non-genotoxicants, 5 have been tested to the above standards (*o*-anthranilic acid, 2-butoxyethanol, pyrene, sodium chloride, sulfisoxazole), while 3 have been tested incompletely (furan, melamine, methylphenidate). All 8 have tested negative in the assay. Of these 8 chemicals, 2-butoxyethanol is of particular significance because it caused marked intravascular lysis of RBCs and induced a strong compensatory erythropoiesis, yet it did not affect *Pig-a* mutant frequencies.

Within the group of agents that have been tested, the Workgroup noted two reports of assays (using 3- and 28-day treatments with aristolochic acids and 28-day treatments with 4-NQO) that were negative for erythrocyte MN induction, but positive for *Pig-a* mutation [27,32]. There also were 3 agents identified by the Workgroup (AZT, hydroxyurea and 5-fluorouracil) that induced micronucleated RETs but did not increase *Pig-a* mutant

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