Received: 10 October 2019 | Revised: 9 March 2020 | Accepted: 13 March 2020

DOI: 10.1002/em.22368

Environmental and Molecular Mutagenesis | Environmental Mutagenesis and Genomics Society | WILEY

RESEARCH ARTICLE

Testing of acetaminophen in support of the international multilaboratory in vivo rat *Pig-a* assay validation trial

Bas-jan van der Leede[1] | Sandy Weiner[2] | Terry Van Doninck[1] | Kathleen De Vlieger[1] | Ann Schuermans[1] | Fetene Tekle[1] | Helena Geys[1] | Marjolein van Heerden[1] | Sandra De Jonghe[1] | Jacky Van Gompel[1]

[1]Janssen Research & Development, Beerse, Antwerp, Belgium

[2]Janssen Research & Development, Spring House, Pennsylvania

Correspondence
Bas-jan van der Leede, Janssen Research & Development Belgium, Turnhoutseweg 30, Beerse, Antwerp 2340, Belgium.
Email: bvdleede@its.jnj.com

Funding information
Janssen Pharmaceutical Companies of Johnson & Johnson; Johnson & Johnson Consumer, Inc, McNeil Consumer Healthcare Division

Accepted by: S. Dertinger

Abstract

Acetaminophen, a nonmutagenic compound as previously concluded from bacteria, in vitro mammalian cell, and in vivo transgenic rat assays, presented a good profile as a nonmutagenic reference compound for use in the international multilaboratory *Pig-a* assay validation. Acetaminophen was administered at 250, 500, 1,000, and 2,000 mg·kg^{-1}·day^{-1} to male Sprague Dawley rats once daily in 3 studies (3 days, 2 weeks, and 1 month with a 1-month recovery group). The 3-Day and 1-Month Studies included assessments of the micronucleus endpoint in peripheral blood erythrocytes and the comet endpoint in liver cells and peripheral blood cells in addition to the *Pig-a* assay; appropriate positive controls were included for each assay. Within these studies, potential toxicity of acetaminophen was evaluated and confirmed by inclusion of liver damage biomarkers and histopathology. Blood was sampled pre-treatment and at multiple time points up to Day 57. *Pig-a* mutant frequencies were determined in total red blood cells (RBCs) and reticulocytes (RETs) as CD59-negative RBC and CD59-negative RET frequencies, respectively. No increases in DNA damage as indicated through *Pig-a*, micronucleus, or comet endpoints were seen in treated rats. All positive controls responded as appropriate. Data from this series of studies demonstrate that acetaminophen is not mutagenic in the rat *Pig-a* model. These data are consistent with multiple studies in other nonclinical models, which have shown that acetaminophen is not mutagenic. At 1,000 mg·kg^{-1}·day^{-1}, Cmax values of acetaminophen on Day 28 were 153,600 ng/ml and 131,500 ng/ml after single and repeat dosing, respectively, which were multiples over that of clinical therapeutic exposures (2.6–6.1 fold for single doses of 4,000 mg and 1,000 mg, respectively, and 11.5 fold for multiple dose of 4,000 mg) (FDA 2002). Data generated were of high quality and valid for contribution to the international multilaboratory validation of the in vivo Rat *Pig-a* Mutation Assay.

KEYWORDS

flow cytometry, glycosyl phosphatidylinositol, in vivo, mutation assay, red blood cells, reticulocytes

1 | INTRODUCTION

The in vivo *Pig-a* assay is a mutation detection assay identified as a useful tool in evaluating the ability of compounds to induce mutations in rodents. *Pig-a* is short for phosphatidylinositol glycan class A gene, located on the X chromosome as a single functional copy per cell, coding for the catalytic subunit of an N-acetyl glucosamine transferase which is involved in biosynthesis of glycosylphosphatidylinositol (GPI) mammalian cell surface anchors proteins. Mutations in the *Pig-a* gene result in loss of the GPI anchors and the proteins that they anchor to the cell exterior surface (Bryce *et al*., 2008). Wild-type peripheral blood erythrocytes can be labeled with a fluorescent antibody against the GPI-anchored protein (CD59) then identified and enumerated by using a flow cytometry. The presence or absence of the GPI-anchored protein (CD59) can be used to discriminate between wild-type and mutant *Pig-a* genes (Miura *et al*., 2008; Dertinger *et al*., 2011; Gollapudia *et al*., 2015).

The *Pig-a* assay is considered a useful option for mechanistic understanding of genotoxicity or to clarify the mode of action of compounds (Dobrovolsky *et al*., 2010; Schuler *et al*., 2011) especially in the risk assessment of in vitro genotoxicants and/or as a follow-up assay to the ICH S2(R1) (ICH, 2011), the ICH M7(R1) (ICH, 2017), and in support of REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) (REACH, 2006) testing. There are other in vivo mutation models available, for example, in vivo *HPRT* (endogenous gene) assay (Strauss and Albertini, 1979; Aidoo *et al*., 1997; Von Tungeln *et al*., 2007) (which is based on ex vivo mitogen stimulation of lymphocytes and resistance to nucleoside 6-thioguanine) and the transgenes-based assays: lacZ and lacI (Gossen *et al*., 1989; Kohler *et al*., 1991; Boerrigter *et al*., 1995; Heddle *et al*., 1995; Manjanatha *et al*., 1998) (which provide information on an organ-specific basis). However, these assays are time-consuming, labor intensive, and/or very costly in comparison to the *Pig-a* assay. A few advantages of using the *Pig-a* assay are that it is sensitive to detect mutagenic agents such as benzo[a] pyrene, chlorambucil, cisplatin, and n-ethyl-n-nitrosourea (Gollapudia *et al*., 2015), it can be used across species/strains as the GPI anchor proteins are highly conserved (Chatterjee and Mayor, 2001; Ferguson *et al*., 2017), the endpoint can be easily incorporated into standard acute or chronic toxicology testing since only peripheral blood is needed to conduct the *Pig-a* assessment (Gollapudia *et al*., 2015), and the *Pig-a* analysis is flow cytometry based (Dertinger *et al*., 2012).

The need to create an Organization for Economic Co-operation and Development (OECD) guidance document for the *Pig-a* assay for potential regulatory acceptance drove the formation of the international multilaboratory *Pig-a* assay validation trial (Dertinger *et al*., 2011; Dertinger and Heflich, 2011; Gollapudia *et al*., 2015). This eventually grew to include 20 international labs that progressed through 4 stages of the trial. Our laboratory was qualified to participate by successfully completing all validation stages. The outcome of this trial was reported by Gollapudia *et al*., 2015.

Acetaminophen was one of the 41 compounds that were selected in support of the international multilaboratory *Pig-a* assay validation trial (Gollapudia *et al*., 2015). Due to its properties of being nonmutagenic (as previously concluded from bacteria, in vitro mammalian cell and in vivo transgenic rat assays) (IARC, 1990; Bergman *et al*., 1996), clastogenic at toxic dose levels (Bergman *et al*., 1996), and noncarcinogenic, acetaminophen presented a good profile as a nonmutagenic reference compound that could be used in the *Pig-a* assay validation.

A series of 3 studies each with progressively longer durations were undertaken as described in detail in Tables 1–3. Acetaminophen was administered by oral gavage once daily for 3 days (3-Day Study), 15 days (2-Week Study), and 29 days (1-Month Study). The 3-Day and 1-Month Studies included assessments of acetaminophen for its potential to induce structural and/or numerical chromosomal aberrations in peripheral blood erythrocytes via inclusion of the micronucleus endpoint and to induce DNA damage in liver cells and peripheral blood cells via inclusion of the comet endpoint. These endpoints were included to confirm the toxicity-induced clastogenic profile of acetaminophen and to investigate the selectivity of the *Pig-a* assay to detect mutagens. Within these studies, acetaminophen was evaluated for liver toxicity that is known to occur at high exposures in rats (Bergman *et al*., 1996) by the inclusion of liver damage biomarkers and histopathology. The 1-Month Study included an assessment of the acetaminophen plasma exposure.

2 | MATERIALS AND METHODS

2.1 | Reagents

Acetaminophen (CAS number 103-90-2) was obtained from Specgx, Raleigh, NC. N-ethyl-N-nitrosourea (ENU; CAS number 759-73-9) and ethyl methanesulfonate (EMS; CAS number 62-50-0) were purchased from Sigma Aldrich, St. Louis, MO. Hydroxypropyl methylcellulose (Methocel F4M Premium) was obtained from Dow Chemical Company, Midland, MI.

All reagents to perform the micronucleus assay were from Rat MicroFlow® PLUS kits (Litron Laboratories, Rochester, NY). Most reagents to perform the *Pig-a* assay were from prototype Rat MutaFlow® kits (Litron Laboratories). Additional supplies included Lympholyte®-Mammal cell separation agent 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. Reagent supplies to perform the comet assay included normal melting point agarose, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-$Na_2.2H_2O$), sodium chloride (NaCl), and sodium hydroxide (NaOH) from Sigma Aldrich; dimethyl sulfoxide (DMSO) and ethanol from Merck, Darmstadt, Germany; Hank's balanced salt solution and Ca2+/Mg2+ free phosphate buffered saline (PBS) and Triton X-100 from ThermoFisher, Waltham, MA; low melting point agarose from Invitrogen; and Tris hydroxymethyl aminomethane from Serva, Heidelberg, Germany.

2.2 | Animals and treatments

All studies were conducted in an AAALAC-approved laboratory of Johnson & Johnson in Belgium. Johnson & Johnson vivarium facilities meet inspection agency standards. All animals were treated humanely and

TABLE 1 Description of dosing groups, assessments, genotoxicity and toxicity in the 3-Day Studies

Type of study	Purpose/endpoints	Treatment[a] ($mg \cdot kg^{-1} \cdot day^{-1}$)	*N* per group	Dosing days	Observation days	Bleeding days	Tissue sampling days	Day of terminal kill
3-Day Study Dose Range Finding Study[b]	Toxicity	250, 500, 1,000, 2,000	3	1–3		NA	NA	4
	Mortality and clinical observations	All			Daily: 1–4			
		All			Time-related: Day 1 at 1, 3, 5 hr; Days 2 and 3 at 1, 3 hr; Day 4 at 3 hr			
	Body weight	All			Days −1, 1, 2, 3: before dosing; Day 4: before terminal kill			
3-Day Study Definitive Study	*Pig-a*[c] and micronucleus (MN)[d] assays	Vehicle 500, 1,000, 2,000	6	1–3		MN: 4 *Pig-a*: −4, 15, 30, 45	NA	45
	Pig-a assay[c]	ENU (40)	3	1–3		*Pig-a*: −4, 15, 30, 45	NA	45
	Comet assay[e] and histopathology[f]	Vehicle 500, 1,000, 2,000	5	1–3		Comet: 3	Comet: 3 Histopathology: 3	3
	Comet assay[e] and histopathology[f]	EMS (200)	3	2–3		Comet: 3	Comet: 3 Histopathology: 3	3
	Mortality and clinical observations	All			Daily: Days 1–45			
		Vehicle, 500, 1,000, 2,000, ENU (40)			Time-related: Day 1 at 0, 1, 3, 5 hr; Days 2 and 3 at 0, 1, 3 hr			
		EMS (200)			Days 2 and 3 at 0, 1, 3 hr			
	Body weight	All			Days 1, 2, 3 before dosing			
		Vehicle, 500, 1,000, 2,000, ENU (40)			Days 8, 15, 22, 29, 36, 43, 46			

Abbreviations: EMS, ethyl methanesulfonate; ENU, N-ethyl-N-nitrosourea; NA, not applicable.
[a]Vehicle, Acetaminophen (250, 500, 1,000 or 2,000), ENU (40) or EMS (200).
[b]The Dose Range Finding Study was conducted prior to initiation of the Definitive Study.
[c]*Pig-a* assay: peripheral blood.
[d]Micronucleus (MN) assay: peripheral blood.
[e]Comet assay: liver and peripheral blood.
[f]Histopathology: liver.

TABLE 2 Description of dosing groups, assessments, genotoxicity and toxicity in the 2-Week Study

Purpose/endpoints	Treatment[a] ($mg \cdot kg^{-1} \cdot day^{-1}$)	*N* per group	Dosing days	Observation days	Bleeding days	Tissue sampling days	Day of terminal kill
Pig-a assay[b], hematology, clinical chemistry	Vehicle 250, 500, 1,000	5	1–15		*Pig-a*: 16 Hematology, clinical chemistry: 16	NA	16
Mortality and clinical observations	All			Daily: 1–16			
				Time-related: Days 1, 8, 15 at −0.5, 0, 1, 2, 4, 6, and 24 hr			
Body weight	All			Daily			
Food consumption	All			Weekly			

Abbreviation: NA, not applicable.
[a]Vehicle, Acetaminophen (250, 500, 1,000 or 2,000).
[b]*Pig-a* assay: peripheral blood.

cared for in accordance with the European (Council Directive, 2007) and Belgium (Belgium Law, 1991) guidelines, and with the principles of euthanasia as stated in the Report of the American Veterinary Medical Association Panel (AVMA, 2001). Male Sprague Dawley rats were purchased from Charles River, Sulzfeld, Germany. Rodents were allowed to acclimate for at least 5 days before use and were 7–8 weeks old at start of treatment. Water and food were available ad libitum throughout acclimation and experimental periods. Experimental animals were group-housed in an environmentally controlled room with temperatures between 20–23°C, relative humidity between 40–70%, and a 12-hr light/dark cycle. General daily clinical observations and once weekly observations at multiple time points were performed for each animal. (Tables 1–3) Body weight and food consumption were monitored throughout the study periods. Rodents were anesthetized by inhalation of an isoflurane/oxygen mixture and euthanized by exsanguination via the carotid artery at the end of the experimental period. The highest treatment level of acetaminophen applied in the studies was the limit dose for genotoxicity studies (1,000 $mg \cdot kg^{-1} \cdot day^{-1}$ (>14 day dosing) and 2,000 $mg \cdot kg^{-1} \cdot day^{-1}$ (<14 day dosing)) according to OECD guidelines (OECD, 2016a; 2016b) as no profound toxicity was observed based on existing and herein reported data.

Animals were randomized into dosing groups based on initial body weight and all treatments were administered by oral gavage. Acetaminophen was formulated as an aqueous suspension in deionized water containing 0.5% w/v Methocel. The vehicle solution was deionized water containing 0.5% w/v Methocel. The ENU and EMS solutions were prepared in PBS (pH 6.0) and 0.9% w/v NaCl, respectively.

Three studies were performed with different durations. Details on the study designs and measured endpoints are presented in Tables 1–3.

2.3 | 3-Day study

In the 3-Day study, there was a dose range finding portion prior to the definitive study in which 4 groups of 3 animals were treated with 250, 500, 1,000 or 2,000 $mg \cdot kg^{-1} \cdot day^{-1}$ of acetaminophen for 3 consecutive days. No recovery phase was included in the dose range finding study. The results of the Dose Range Finding Study supported dosing up to 2,000 $mg \cdot kg^{-1} \cdot day^{-1}$ in the definitive portion of the study as there were no acetaminophen treatment-related mortality or clinical abnormalities observed. The 2,000 $mg \cdot kg^{-1} \cdot day^{-1}$ top dose of acetaminophen was selected as it is considered the limit in vivo dose per OECD TG474 (OECD, 2016a) and because it is a dose high enough to induce hepatotoxicity, but was not lethal, as shown in previous studies (Boyd and Bereczky, 1966; Abdel-Zaher *et al*., 2008). In the definitive portion of the study 4 groups of 6 animals were treated with 0, 500, 1,000 or 2,000 $mg \cdot kg^{-1} \cdot day^{-1}$ of acetaminophen for 3 consecutive days. A separate group of 3 animals was treated with 40 $mg \cdot kg^{-1} \cdot day^{-1}$ of ENU (positive control for the *Pig-a* endpoint) for 3 consecutive days. The second treatment was administered approximately 24 hr after the first treatment while the third treatment was administered 21 hr after the second treatment. A subsequent 42-day recovery phase was included. For the comet assay in the definitive portion of the study, 4 groups of 5 animals were treated with 0, 500, 1,000, or 2,000 $mg \cdot kg^{-1} \cdot day^{-1}$ of acetaminophen for 3 consecutive days at an approximately 24-hr interval between the first and second dose and 21 hr between the second and third dose. A separate group of 3 animals was treated with 200 $mg \cdot kg^{-1} \cdot day^{-1}$ of EMS (positive control for the comet endpoint) for the 2 last consecutive days, approximately 21 hr apart.

2.4 | 2-Week study

A 2-week study was conducted as a bridging study between the 3-Day and 1-Month Studies to identify the overall (to animal and endpoint) tolerability to the high doses of acetaminophen. In the 2-Week Study, 4 groups of 5 animals were treated with 0, 250, 500 or 1,000 $mg \cdot kg^{-1} \cdot day^{-1}$ of acetaminophen for 15 consecutive days;

TABLE 3 Description of dosing groups, assessments, genotoxicity, toxicity, and plasma acetaminophen exposure in the 1-Month Study

Type of study	Purpose/endpoints	Treatment[a] ($mg \cdot kg^{-1} \cdot day^{-1}$)	*N* per group	Dosing days	Observation days	Bleeding days	Tissue sampling days	Day of terminal kill
1-Month Study Genetic Toxicity	*Pig-a*[b], MN[c] and comet assays[d], hematology, clinical chemistry and histopathology[e]	Vehicle 250, 500, 1,000	6	1–29		*Pig-a*: –1, 15, 29 MN: 4, 29 Comet: 29 hematology, clinical chemistry: 29	Comet: 29 histopathology: 29	29
	Pig-a assay[b]	ENU (40)	3	1–3		*Pig-a*: –1, 15, 29	NA	29
	Comet assay[c] and histopathology[d]	EMS (200)	3	28–29		Comet: 29	Comet: 29 Histopathology: 29	29
	Mortality and clinical observations	All			Daily: 1–29			
					Time-related: Days 1, 8, 15, 22, 28 at –0.5, 0, 1, 2, 4, 6, 24 hr			
	Body weight	All			Daily			
	Food consumption	All			Weekly			
1-Month Study Genetic Toxicity Recovery	*Pig-a* assay[b] and histopathology[e]	Vehicle 1,000	6	1–29		*Pig-a*: –1, 15, 29, 57	Histopathology: 57	57
	Mortality and clinical observations	All			Daily: 1–57			
					Time-related: Days 1, 8, 15, 22, 28 at –0.5, 0, 1, 2, 4, 6, 24 hr			
	Body weight	All			Daily during dosing period; Days 36, 43, 50, 57 during recovery period			
	Food consumption	All			Weekly			
1-Month Study Toxicity	Toxicity, hematology, clinical chemistry and histopathology[e]	Vehicle 1,000	10	1–7 (*N* = 5) 1–28 (*N* = 5)		Hematology, clinical chemistry: 2, 8, 16, 29	Histopathology: 8, 29	8 (*N* = 5) 29 (*N* = 5)
	Mortality and clinical observations	All			Daily: 1–29			
					Time-related: Days 1, 8, 15, 22, 28 at			

TABLE 3 (Continued)

Type of study	Purpose/endpoints	Treatment[a] (mg·kg^{-1}·day^{-1})	N per group	Dosing days	Observation days	Bleeding days	Tissue sampling days	Day of terminal kill
					–0.5, 0, 1, 2, 4, 6, 24 hr			
	Body weight	All			Daily			
	Food consumption	All			Weekly			
1-Month Study Toxicokinetics	Toxicokinetics (TK) and histopathology[e]	1,000	5	1–28		TK: 1 and 28 at 0.5, 1, 2, 4, 7 hr; 2 and 29 at 24 hrs	Histopathology: 29	29
	Mortality and clinical observations	All			Daily			
	Body weight	All			Daily			

Abbreviations: EMS, ethyl methanesulfonate; ENU, N-ethyl-N-nitrosourea; NA, not applicable; TK, toxicokinetics.

[a]Vehicle, Acetaminophen (250, 500, 1,000 or 2,000), ENU (40) or EMS (200).

[b]*Pig-a* assay: peripheral blood.

[c]Micronucleus (MN) assays: peripheral blood.

[d]Comet assay: liver and peripheral blood.

[e]Histopathology: liver.

treatments were administered approximately 24 hr apart. This study did not include a recovery phase.

2.5 | 1-Month study

In the 1-Month study, 4 groups of 6 animals were treated with 0, 250, 500 or 1,000 mg·kg^{-1}·day^{-1} of acetaminophen for 29 consecutive days (genetic toxicity group). The daily treatments were administered on approximately 24-hr intervals, while the last treatment was administered 21 hr after the previous treatment. An additional group of 3 animals was treated with 40 mg·kg^{-1}·day^{-1} of ENU (positive control for the *Pig-a* endpoint) only for the first 3 consecutive days. For the comet assay, an additional group of 3 animals was treated with 200 mg·kg^{-1}·day^{-1} of EMS (positive control for the comet endpoint) only for the last 2 consecutive days, with dosing at approximately 21-hr interval. Two additional groups of 5 animals were included to evaluate general toxicity endpoints after 7 or 28 days of treatment (i.e., toxicity group); these groups were treated with 0 or 1,000 mg·kg^{-1}·day^{-1} of acetaminophen in parallel with the main group. Two additional groups of 6 animals treated for 29 days with 0 or 1,000 mg·kg^{-1}·day^{-1} of acetaminophen were included. These animals were monitored and sampled during the treatment period and at the end of a 28-day recovery period (ie, genetic toxicity recovery group). The *Pig-a* and micronucleus assays employing hematopoietic cells requires exposure of blood and bone marrow to adequately test chemicals; therefore, measurement of acetaminophen plasma levels was included. A group of 5 animals treated with 1,000 mg·kg^{-1}·day^{-1} of acetaminophen was included to determine systemic exposure to acetaminophen (i.e., toxicokinetic satellite group).

2.6 | *Pig-a* assay

Peripheral blood samples were obtained by puncture of the tail or sublingual veins. Drops of blood were collected into an empty 1 ml lithium-heparin-coated tube. Immediately following sampling, 80 μl of blood sample was transferred into a 1 ml tube containing 100 μl of anticoagulant solution (Prototype Rat *Pig-a* Mutation Assay Kit) and gently mixed. Blood samples were stored refrigerated until further processing and analysis by flow cytometry. Peripheral blood samples were processed for *Pig-a* mutant frequency according to reagents and instructions that accompanied the Prototype Rat *Pig-a* Mutation Assay Kit. Erythrocytes were separated from platelets and leukocytes by centrifugation with Lympholyte® solution. Enriched erythrocytes were incubated with anti-CD59-PE and anti-CD61-PE antibodies. Following a wash step, samples were incubated with anti-PE paramagnetic particles. A small portion of each sample was incubated with the cell permeable nucleic acid dye SYTO® 13 and fluorescent latex particles (Counting Beads) and subsequently analyzed on a flow cytometer to capture cell to Counting Bead ratio (pre-column analysis). The major portion of each sample was passed through a ferromagnetic column placed in a magnetic field to deplete each sample of wild-type

cells; mutant cells freely pass through the column. The eluate was concentrated by centrifugation, incubated with the cell permeable nucleic acid dye SYTO® 13 and fluorescent latex particles (Counting Beads), and subsequently analyzed on a flow cytometer to capture mutant cell to Counting Bead ratio (post-column analysis). The samples were analyzed on a BD FACS Calibur instrument with a stop time of 2 and 6 min for pre- and post-column analysis, respectively. Before analyzing the experimental samples, instrument settings were configured with the Instrument Calibration Standard as described in the user manual of the Prototype Rat *Pig-a* Mutation Assay Kit. From the pre- and post-column analyses, the reticulocyte (RET) percentage, mutant phenotype erythrocyte (CD59-negative red blood cell [RBC]), and mutant phenotype RET (CD59-negative RET) frequencies were calculated. Upon analysis of the pretreatment blood samples, rats demonstrating an elevated background mutant phenotype erythrocyte or RET frequency were excluded from study dosage group assignment. The evaluation criteria for a positive response applied were (a) a least 1 of the dose groups exhibits a statistically significant increase in mutant phenotype RET or erythrocyte frequencies relative to concurrent vehicle group and (b) the increase in mutant phenotype RET or erythrocyte frequencies is dose-related when evaluated with an appropriate trend test.

2.7 | Micronucleus assay

Peripheral blood samples were obtained by puncture of the tail or sublingual veins and drops of blood (approximately 50 or 150 μl) were collected into a 1 or 2 ml tube containing 450 or 850 μl anticoagulant (Rat MicroFlow® PLUS Kit), respectively. Immediately following sampling, the tube was mixed gently, and 180 μl of anticoagulated blood was added to 2 ml ultracold methanol. Each blood sample was fixed in duplicate and stored in the ultrafreezer until analysis by flow cytometry. Fixed blood samples were processed using the Rat MicroFlow®PLUS Kit. The fixed blood samples were washed and incubated simultaneously with RNase A, anti-CD71-FITC, and anti-CD61-PE antibody for 30 min on melting ice protected from light, and subsequently for 30 min at room temperature protected from light. Immediately before analysis, the samples were resuspended in an appropriate amount of staining solution containing propidium iodide (PI) to obtain an adequate flow rate on the flow cytometer. The samples were analyzed using a BD FACS Calibur instrument. The flow cytometer photomultiplier tube and compensation were adjusted using biostandards to optimize the flow cytometer for micronucleus scoring of peripheral RBCs according to the instructions in the user manual of the Rat MicroFlow®PLUS Kit. The number of micronucleated and nonmicronucleated RETs, and the number of micronucleated and nonmicronucleated normochromatic erythrocytes (NCEs) was recorded by the flow cytometer. Data acquisition stopped when 20,000 RETs had been collected. Before analyzing the experimental samples, positive and negative control samples (provided within the Rat MicroFlow®PLUS Kit) were analyzed. The percentage of micronucleated RETs in the negative and positive control samples were within the respective ranges for these samples demonstrating that the flow cytometer settings were optimal for micronucleus detection in the experimental samples. The evaluation criteria for a positive response applied were according to OECD TG 474 guidance (OECD, 2016a).

2.8 | Comet assay

Blood samples were collected from the carotid artery in 4 ml sodium heparin-coated BD Vacutainer tubes. Immediately following sampling, the tubes were mixed and placed on ice until preparation of comet slides.

A standardized portion of the left lateral liver lobe was excised and washed in cold mincing buffer (Hank's balanced salt solution with 20 mM EDTA-Na2 and 10% DMSO; pH 7.5) to remove excess blood. The liver portion was minced with a pair of fine scissors to release the cells. This tissue processing was conducted under dim yellow room lighting and on a cold surface in order to prevent induction of additional DNA damage. The cell suspension was strained through a cell strainer to remove lumps and then used to prepare the comet slides. Another section of the left lateral liver lobe and the right medial lobe was placed in 10% buffered formalin and processed for histopathological examination.

From each cell suspension (blood or liver), 3 replicate samples were prepared, and each slide contained 2 different samples (2 × 75 μl). From each anticoagulated blood or liver sample, an aliquot of 20 or 25 μl, respectively, was mixed with 300 μl low-melting point agarose (0.5% w/v PBS). The cell/agarose suspension was applied to glass microscope slides, previously coated with normal melting agarose (1% w/v in water) and a coverslip was placed on top. The slides were set on ice for approximately 15 min to allow the gel to solidify. Then the coverslips were removed, and slides were submerged in a cold lysing solution (100 mM EDTA-Na2, 2.5 M NaCl, 10 mM Tris hydroxymethyl aminomethane, pH 10.0; 1% Triton X-100 and 10% DMSO added just before use) and remained in this solution at least overnight at 2–8°C under a light proof condition. After cell lysis, the slides were washed with neutralization buffer (0.4 M Tris hydroxymethyl aminomethane; pH 7.5) and randomly placed in the electrophoresis chamber. The unwinding solution was added (300 mM NaOH and 1 mM EDTA-Na2; pH > 13) and poured until the surfaces of the slides were completely covered. The slides were left to unwind for 20 min in the dark. Thereafter, electrophoresis was conducted in the same buffer for 30 min at 0.7 V/cm, at approximately 4–8°C, at approximately 300 mA. After electrophoresis, the slides were removed from the chamber and washed with neutralization buffer (0.4 M Tris hydroxymethyl aminomethane; pH 7.5) for at least 5 min. Then the gels were dehydrated with 100% ethanol for at least 5 min, air dried, and stored at room temperature protected from dust and humidity. The slides were stained with ethidium bromide (20 μg/ml) prior to scoring. Coded slides were scored blindly. Two replicate samples per animal were used for scoring. Fifty randomly selected, nonoverlapping cells per sample were scored for DNA damage using a semi-automated image analysis system coupled to a fluorescence

microscope. DNA damage was assessed using the software system by measuring % tail intensity. The percentage of "hedgehog" cells (a morphology indicative of highly damaged cells which can be associated with severe genotoxicity, necrosis, or apoptosis (OECD TG 489); also known as "clouds" or "ghosts") among 100 scored nuclei per animal was recorded. The median of 100 counts of % tail intensity was determined for each animal in each treatment group. The mean and standard deviation of the median values for % tail intensity were calculated for each treatment group. The evaluation criteria for a positive response applied were according to OECD TG 489 guidance (OECD, 2016b).

2.9 | Clinical and anatomic pathology assessments

In addition to the genotoxicity assessments, hematology, clinical chemistry, and necropsy with macroscopic evaluation of tissues, liver weight measurement, and histopathology of the liver, kidney, and gross findings were conducted. (Tables 1–3) Blood and tissue samples were collected at necropsy. Blood samples were analyzed for standard hematological (Siemens ADVIA®2,120 Hematology Analyzer) and clinical pathological (Roche Diagnostics Cobas® 6,000 c501 Analyzer) parameters. Tissue samples were fixed in 10% buffered formalin, trimmed, embedded in paraffin blocks, and sectioned. Slides were stained with hematoxylin–eosin and examined by microscopy.

2.10 | Toxicokinetics

Peripheral blood samples were collected by puncture of the tail vein and collected in K3EDTA tubes. Within 1 hr of blood sampling, centrifugation of blood samples to allow plasma separation was initiated. Blood samples were centrifuged at room temperature for 10 min at 1900*g*. Immediately thereafter, plasma was separated, transferred into a second tube, and stored in the freezer within 2 hr after the start of centrifugation. Plasma samples of acetaminophen-treated groups were analyzed individually for acetaminophen levels using a qualified LC–MS/MS method. The samples were subjected to a selective sample cleanup, followed by HPLC-MS/MS.

2.11 | Statistical analyses

Statistically significant differences between each acetaminophen treatment group and positive control group against the vehicle group were assessed for the general toxicity parameters mortality, clinical observations, and histopathology by Fisher Exact Probability test; body weight and weight gain, hematology and clinical chemistry by Mann–Whitney *U* test; organ weights by Dunn's test with Bonferoni-Holm correction. All tests were performed at a significance level of 5% or less.

For the *Pig-a* assay, statistical analyses were performed on the mutant cell frequencies CD59-negative RBC and CD59-negative RET per 10^6 and RET percent (%RET) as described in Avlasevich et al. (Avlasevich *et al*., 2018). Accordingly, CD59-negative RBC and CD59-negative RET per 10^6 frequencies were log(10) transformed with a 0.1 offset added to each frequency prior to log transformation. Each time point was studied separately, where the effect of treatment on these untransformed RET, CD59-negative RBC, and CD59-negative RET data were compared to vehicle using Dunnett's multiple comparison *t* tests in the context of a one-way analysis of variance. Significance was evaluated using a one-tailed test for increases relative to vehicle. Similar analyses were performed on the %RET data as well; however, tests were then performed on the raw (untransformed) data and two-tailed. When Dunnett's test indicated a significant increase to %RET, CD59-negative RBC, or CD59-negative RET, the data for the endpoint and time point in question were evaluated for a dose-related linear trend. Whenever there is a positive control group, it was compared to the vehicle group by means of a two-sided *t* test for %RET and one-sided *t* test for the log-transformed frequencies of CD59-negative RBC and CD59-negative RET.

For the micronucleus test, statistical analyses were performed on the percentages of RETs and micronucleated RETs. Data were checked for non-normality by applying the Shapiro–Wilk test (Shapiro and Wilk, 1964) which checks whether a random sample of values follows a normal distribution. When data were not normally distributed, the percentage of RETs was square-root transformed and the percentage of micronucleated RETs was log-transformed and subsequently rechecked for non-normality using the Shapiro–Wilk test. When data were normally distributed, homogeneity of variances was assessed through Levene's test (Levene, 1960). When data were either not normally distributed or homogeneity of variances could be rejected, then Dunn's test was applied for comparison of the acetaminophen treatment groups to the vehicle group (Dunn, 1964). Otherwise, Dunnett's test was applied two-sided for the percentage of RETs and one-sided for the percentage of micronucleated RETs. In the case of a statistically significant difference between 1 or more acetaminophen treatment groups and the vehicle group, a Jonckheere Terpstra trend test (Jonckheere, 1954) was performed to assess a potential dose–response trend.

For the comet assay, statistical analyses were performed on % tail intensity in alignment with the recommendations described by Bright *et al*. (2011). The individual nucleus data were log transformed (a small positive value, that is, 0.001, was added to each measurement to avoid taking the logarithm of zero). Next, the geometric mean was calculated per replicate slide of each animal. In order to deal with the hierarchical nature of the data (several samples from the same animal), a mixed effects model (Verbeke and Molenberghs, 2000) was fitted to the data with animal as random effect and treatment and sample as fixed effects. In case there was no sample effect, the model is reduced to a mixed effects model with only treatment as fixed effect. The denominator degrees of freedom for tests of fixed effects were calculated using the Kenward-Rogers method. Post-hoc right one-sided pairwise comparisons were performed for each acetaminophen treatment group versus the vehicle group. In the case of a statistically significant difference between 1 or more acetaminophen treatment groups and the vehicle group, a one-sided linear trend test was performed to assess a potential dose–response trend. The positive control group was compared to the vehicle group by means of a one-sided *t* test.

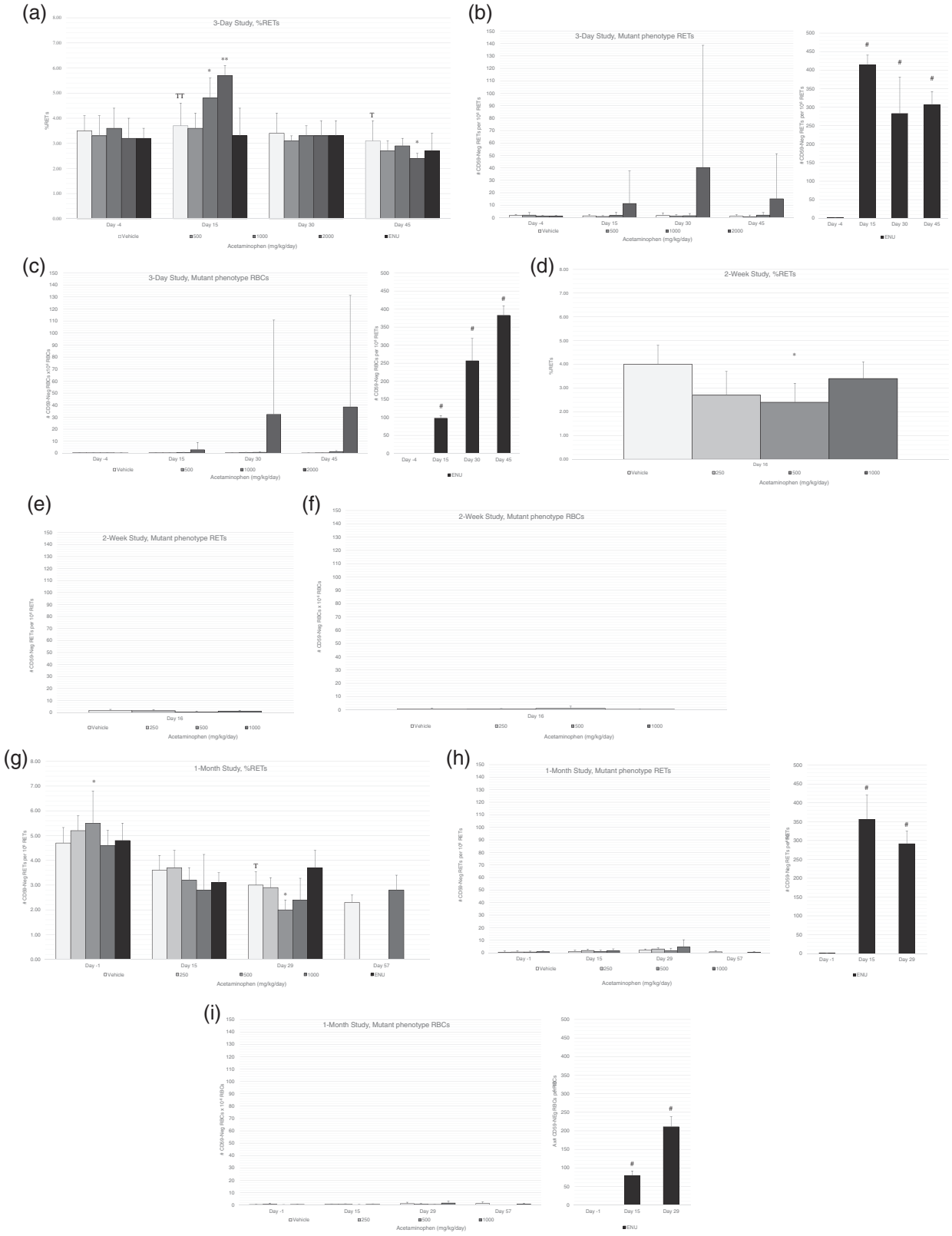

FIGURE 1 Pig-a mutation assay results in rats treated with acetaminophen in the 3-Day, 2-Week, and 1-Month Studies. (A–C) 3-Day Study, (D–F): 2-Week Study and (G–I): 1-Month Study. Data given as Mean ± *SD* for %RET, mutant phenotype RET frequency and mutant phenotype RBC frequency. *Pig-a* data from the 1-Month Study on Days −1, 15 and 29 from the main and recovery animals of the vehicle and 1,000 $mg \cdot kg^{-1} \cdot day^{-1}$ groups were combined in the statistical analysis. ENU was used as positive control. Significance versus vehicle by two-sided Dunnett's test: $^{*}p < .05$; $^{**}p < .001$; significance by two-sided linear trend test: $^{T}p < .05$; $^{TT}p < .001$; significance versus vehicle by one-sided *t* test: $^{\#}p < .001$

3 | RESULTS

Acetaminophen was administered to male Sprague Dawley rats once daily by oral gavage at 250, 500, 1,000, or 2,000 mg·kg^{-1}·day^{-1} in a series of 3 studies, each with progressive longer durations, 3 days, 2 weeks, and 1 month with a recovery group. The toxicity assessments performed are described in the sections below.

3.1 | General toxicity assessments

3.1.1 | Clinical observations

No mortality occurred in the 3-Day and 2-Week Studies. In the 1-Month Study, 1 rat treated with 1,000 mg·kg^{-1}·day^{-1} was found dead during the recovery period on study Day 50 without preceding clinical signs of toxicity or evident cause of death based on histological examination. At necropsy, this animal showed small testes and epididymides with abscesses on the preputial glands. Liver histology showed no treatment-related findings. The cause of death was not evident.

Clinical abnormalities were limited to temporary narrowing of palpebral fissure at acetaminophen treatments of ≥1,000 mg·kg^{-1}·day^{-1} in the 3-Day Study and to chromodacryorrhea "bloody tears" at 1,000 mg·kg^{-1}·day^{-1} in only 1 out of 22 animals in the 1-Month Study.

Dose-related body weight loss up to 6.5% compared to pre-treatment was noted at ≥500 mg·kg^{-1}·day^{-1} during the treatment period in the definitive group of the 3-Day Study. In the 2-Week and 1-Month Studies, transient loss of body weight up to 10% compared to pre-treatment was observed at 1,000 mg·kg^{-1}·day^{-1} after 7 days of dosing. Upon further treatment, the body weight had almost recovered to pretreatment values after 14 days of treatment.

3.1.2 | Hematology

In the 2-Week Study, there was a statistically significant and dose-related reduction in white blood cells (WBC), neutrophils, and lymphocytes at acetaminophen at dose levels of ≥500 mg·kg^{-1}·day^{-1} at the end of the treatment period. (Appendix 1).

In the 1-Month Study, an acetaminophen-related effect on several RBC parameters hemoglobin, mean cell hemoglobin concentration (MCHC), mean cell hemoglobin (MCH), mean cell volume (MCV), and RETs (Appendix 1) were observed mainly at 1,000 mg·kg^{-1}·day^{-1}. The observed changes were all slight, transient, and values were comparable to vehicle-treated animals toward the end of the dosing period, suggesting an adaptive response. Although RBC counts were comparably reduced from Day 8 onwards, the strongest reduction in hematocrit and hemoglobin was detected on Day 8 while the MCV was increased from Day 16 onwards. In addition, RETs were also clearly reduced on Day 8 after which their levels rose again to a marginal elevation on Day 16. Although total WBC count was not affected, neutrophils were moderately increased in all acetaminophen-treated groups, but there was no evidence of a dose-related effect. When compared to their respective vehicle group, neutrophils and lymphocytes were most affected on Day 2.

3.1.3 | Clinical chemistry

In the 2-Week Study, statistically significant elevations in calcium, total protein, albumin, cholesterol, and alanine aminotransferase (ALT), and reduction in creatinine were observed for acetaminophen at a dose level of 1,000 mg·kg^{-1}·day^{-1}. In addition, aspartate aminotransferase (AST) was reduced at 250 and 500 mg·kg^{-1}·day^{-1}. (Appendix 2).

In the 1-Month Study, an acetaminophen-related effect on liver function was noted at a dose level of 500 mg·kg^{-1}·day^{-1} as marginal to marked and statistically significant at 1,000 mg·kg^{-1}·day^{-1} elevations in bilirubin, AST, ALT, glutamate dehydrogenase (GLDH), sorbitol dehydrogenase (SDH), and total bile acids (Appendix 2), at 1 or more time points. The marginal to slight increases in total protein, albumin, and cholesterol at 1,000 mg·kg^{-1}·day^{-1} at 1 or more time points also suggested a potential minor effect on liver function. In addition, a slight increase in calcium was detected at 1,000 mg·kg^{-1}·day^{-1}.

3.1.4 | Histopathology

In the 3-Day Study, treatment with acetaminophen at a dose level of 500 mg·kg^{-1}·day^{-1} resulted in centrilobular single-cell necrosis and chronic centrilobular inflammation in the liver of 2/5 male rats. (Appendix 3) At 1,000 and 2,000 mg·kg^{-1}·day^{-1}, centrilobular necrosis with chronic centrilobular inflammation was observed in the liver of all rats. In the majority of the rats, centrilobular hemorrhages with erythrophagocytosis, and ballooning degeneration of adjacent hepatocytes were also observed. Multifocal (degenerated) granulocytic infiltrates were occasionally noted in these necrotic centrilobular areas. This was associated with an increase in midzonal mitotic figures and sometimes with small basophilic periportal hepatocytes, both indicative for regeneration.

In the 1-Month Study, daily oral acetaminophen at a dose level of 1,000 mg·kg^{-1}·day^{-1} for 1 week or 1 month resulted in a slight increase in liver weight and gastric distention (both time points). At histology, minimal to slight liver changes were observed in the centrilobular region of 5/5 rats after 1 week and 9/16 rats after 1 month. The affected rats presented 1 or more of the following findings: centrilobular chronic inflammation or fibrosis (with some bridging), apoptosis/single cell necrosis, brown pigmented macrophages, hepatocellular vacuolization, hydropic degeneration, and/or nonzonal focal or single-cell necrosis. Centrilobular regenerative findings were noted in some rats (dark/irregular/binucleated hepatocytes after 1 week and centrilobular dense staining cytoplasm after 1 month).

After a 1-month recovery period (only liver data collected), liver weight was normalized and only minimal remnants of treatment-related changes were noted in 2/6 rats (focal centrilobular fibrosis and focal presence of brown pigmented macrophages), indicating almost complete recovery.

TABLE 4 *Pig-a* mutation assay results in rats treated with acetaminophen in the 3-Day Study

Treatment ($mg \cdot kg^{-1} \cdot day^{-1}$)	Number of animals	%RETs mean ± *SD*	Number of CD59-Neg RBCs per 10^6 RBCs mean ± *SD*	Number of CD59-Neg RETs per 10^6 RETs mean ± *SD*
Day −4				
Vehicle	6	3.5 ± 0.6	0.4 ± 0.2	1.7 ± 0.9
500	6	3.3 ± 0.8	0.4 ± 0.2	2.0 ± 2.1
1,000	6	3.6 ± 0.8	0.4 ± 0.2	1.2 ± 0.5
2,000	6	3.2 ± 0.8	0.3 ± 0.1	1.2 ± 0.6
ENU[a], 40	3	3.2 ± 0.4	0.4 ± 0.1	1.6 ± 0.7
Day 15				
0	6	3.7 ± 0.9^{TT}	0.4 ± 0.3	1.4 ± 1.1
500	6	3.6 ± 0.6	0.4 ± 0.2	0.9 ± 0.7
1,000	6	4.8 ± 0.8*	0.6 ± 0.2	1.8 ± 2.4
2,000	6	5.7 ± 0.4**	2.9 ± 6.1	11.3 ± 26.2
ENU[a], 40	3	3.3 ± 1.1	97.7 ± 7.2$^{\#}$	414.2 ± 6.5$^{\#}$
Day 30				
Vehicle	6	3.4 ± 0.8	0.4 ± 0.3	1.7 ± 2.0
500	6	3.1 ± 0.2	0.5 ± 0.3	1.2 ± 1.2
1,000	6	3.3 ± 0.4	0.8 ± 0.4	1.5 ± 1.8
2,000	6	3.3 ± 0.6	32.4 ± 78.4	40.3 ± 98.3
ENU[a], 40	3	3.3 ± 0.6	256.7 ± 62.0$^{\#}$	282.7 ± 77.4$^{\#}$
Day 45				
Vehicle	6	3.1 ± .0.8^{T}	0.3 ± 0.1	1.1 ± 1.2
500	6	2.7 ± 0.4	0.4 ± 0.2	0.7 ± 1.3
1,000	6	2.9 ± 0.3	1.1 ± 1.1	2.0 ± 2.1
2,000	6	2.4 ± 0.2*	38.5 ± 92.8	15.1 ± 36.2
ENU[a], 40	3	2.7 ± 0.7	382.7 ± 26.3$^{\#}$	305.9 ± 59.5$^{\#}$

Note: Significance versus vehicle by two-sided Dunnett's test: $^{*}p < .05$; $^{**}p < .001$; significance by two-sided linear trend test: $^{T}p < .05$; $^{TT}p < .001$; significance versus vehicle by one-sided *t* test: $^{\#}p < .001$.
Abbreviations: RBCs, red blood cells; RETs, reticulocytes; *SD*, standard deviation.
[a]ENU was used as positive control.

TABLE 5 *Pig-a* mutation assay results in rats treated with acetaminophen in the 2-Week Study

Treatment ($mg \cdot kg^{-1} \cdot day^{-1}$)	Number of animals	%RETs mean ± *SD*	Number of CD59-Neg RBCs per 10^6 RBCs mean ± *SD*	Number of CD59-Neg RETs per 10^6 RETs mean ± *SD*
Day 16				
Vehicle	5	4.0 ± 0.8	0.8 ± 0.4	1.8 ± 1.6
250	5	2.7 ± 1.0	0.7 ± 0.3	1.4 ± 1.1
500	5	2.4 ± 0.8*	1.1 ± 1.7	0.4 ± 0.4
1,000	5	3.4 ± 0.7	0.5 ± 0.3	1.1 ± 0.9

Note: Significance versus vehicle by two-sided Dunnett's test: $^{*}p < .05$.
Abbreviations: RBCs, red blood cells; RETs, reticulocytes; *SD*, standard deviation.

3.2 | Genotoxicity assessments

3.2.1 | *Pig-a* assay

In the 3-Day Study, treatment with acetaminophen at dose levels of 1,000 and 2,000 $mg \cdot kg^{-1} \cdot day^{-1}$ led to a temporary increase in %RETs on Day 15. (Figure 1 and Table 4). Stimulated erythropoiesis likely explains this observation as a marked reduction in %RETs was observed at 1,000 and 2,000 $mg \cdot kg^{-1} \cdot day^{-1}$ in the micronucleus test evaluated on Day 4. No statistically significant or dose-related increase in the frequency of mutant phenotype RETs (CD59-negative) and RBCs (CD59-negative) was observed at any sampling time (Days

TABLE 6 *Pig-a* mutation assay results in rats treated with acetaminophen in the 1-Month Study

Treatment ($mg \cdot kg^{-1} \cdot day^{-1}$)	Number of animals	%RETs mean ± *SD*	Number of CD59-Neg RBCs per 10^6 RBCs mean ± *SD*	Number of CD59-Neg RETs per 10^6 RETs mean ± *SD*
Day −1				
Vehicle	12	4.7 ± 0.6	0.4 ± 0.2	0.4 ± 0.3
250	6	5.2 ± 0.6	0.7 ± 0.8	0.6 ± 0.6
500	6	5.5 ± 1.3*	0.3 ± 0.1	0.6 ± 0.7
1,000	12	4.6 ± 0.6	0.5 ± 0.3	0.7 ± 0.7
ENU[a], 40	3	4.8 ± 0.7	0.6 ± 0.3	0.5 ± 0.5
Day 15				
Vehicle	12	3.6 ± 0.6	0.6 ± 0.3	1.0 ± 1.0
250	6	3.7 ± 0.7	0.6 ± 0.4	1.8 ± 2.8
500	6	3.2 ± 0.5	0.3 ± 0.2	1.0 ± 1.0
1,000	12	2.8 ± 1.4	0.6 ± 0.4	1.6 ± 1.5
ENU[a], 40	3	3.1 ± 0.4	79.5 ± 12.3#	356.2 ± 64.4#
Day 29				
Vehicle	12	3.0 ± 0.5T	1.1 ± 1.2	2.0 ± 2.0
250	6	2.9 ± 0.4	0.9 ± 0.3	3.0 ± 2.2
500	6	2.0 ± 0.4*	0.5 ± 0.1	1.7 ± 1.9
1,000	12	2.4 ± 0.9	1.6 ± 1.6	4.5 ± 5.9
ENU[a], 40	3	3.7 ± 0.7	211.0 ± 26.9#	291.4 ± 33.7#
Day 57[b]				
Vehicle	6	2.3 ± 0.3	1.2 ± 1.3	0.7 ± 0.6
1,000	6	2.8 ± 0.6	0.9 ± 0.5	0.5 ± 0.4

Note: Significance versus vehicle by two-sided Dunnett's test: *$p < .05$; significance by two-sided linear trend test: T$p < .05$; significance versus vehicle by one-sided *t* test: #$p < .001$.
Abbreviations: RBCs, red blood cells; RETs, reticulocytes; *SD*, standard deviation.
[a]ENU was used as positive control.
[b]Only Vehicle and 1,000 $mg \cdot kg^{-1} \cdot day^{-1}$ were included in this Day 57 recovery group and hence available for *Pig-a* analysis.

15, 30, and 45). Animals of the positive control group showed pronounced increases in mutant phenotype RETs and RBCs frequencies at the post-treatment sampling times as a result of the mutagenic activity of ENU.

In the 2-Week Study, treatment-related decreases were observed in the %RETs on Day 16 in all acetaminophen treatment groups. (Figure 1 and Table 5) No increase in mutant phenotype RET (CD59-negative RETs) or RBC (CD59-negative RBCs) frequencies was observed in the acetaminophen groups on Day 16. The purpose of this study was to identify the overall (animal and end point) tolerability to high doses of acetaminophen for treatment longer than 3 days in order to select appropriate dose levels for the 1-Month Study. Although this study did not fulfill the minimal requirements to assess *Pig-a* mutation as per International Workshops on Genotoxicity Testing (IWGT) recommendations (Gollapudia *et al*., 2015), detection of mutants was demonstrated by using mutant mimic standards resulting in observed *Pig-a* mutant frequencies in the acetaminophen groups comparable to concurrent vehicle group.

In the 1-Month Study, treatment with acetaminophen at dose levels of 500 and 1,000 $mg \cdot kg^{-1} \cdot day^{-1}$ resulted in slight decreases in %RETs on Day 29 but by Day 57, the %RET had recovered (only 1,000 $mg \cdot kg^{-1} \cdot day^{-1}$ evaluated). (Figure 1 and Table 6). No statistically significant or dose-related increase in the mutant phenotype RET (CD59-negative RETs) or RBC (CD59-negative RBCs) frequencies was observed at any sampling time (Days 15, 29, and 57). Animals of the positive control group showed pronounced increases in mutant phenotype RET (CD59-negative RETs) or RBC (CD59-negative RBCs) frequencies at all the post-treatment sampling times.

3.2.2 | Micronucleus assay

In the 3-Day Study, treatment with acetaminophen-at dose levels of 500 and 1,000 $mg \cdot kg^{-1} \cdot day^{-1}$ did not result in a biologically relevant or statistically significant increase in the percent of micronucleated RETs (%MN-RETs). (Table 7) In addition, there was no evidence for a dose-related increase in the %MN-RETs. The 2,000 $mg \cdot kg^{-1} \cdot day^{-1}$ treatment could not be evaluated for micronucleus induction due to severe reduction in the %RET. The loss of the data from the 2,000 $mg \cdot kg^{-1} \cdot day^{-1}$ dose group due to toxicity observed in the peripheral blood resulted in the conclusions of this study as being an exception to the OECD 474 guidance (OECD, 2016a).

TABLE 7 Micronucleus assay results in rats treated with acetaminophen in the 3-Day and 1-Month Studies

Treatment (mg·kg^{-1}·day^{-1})	Number of animals	Sampling time	%RETs mean ± *SD*	%MN-RETs mean ± *SD*
3-Day Study				
Vehicle	6	Day 4	1.51 ± 0.30†	0.11 ± 0.30
500	6	Day 4	0.89 ± 0.68	0.12 ± 0.05
1,000	6	Day 4	0.08 ± 0.08*	0.21[a]
2,000	6	Day 4	0.03 ± 0.04**	NR
1-Month Study				
Vehicle	6	Day 4	2.75 ± 1.13†	0.14 ± 0.05
250	6	Day 4	2.89 ± 1.89	0.14 ± 0.03
500	6	Day 4	1.80 ± 1.98	0.18 ± 0.07
1,000	6	Day 4	0.03 ± 0.02*	NR
Vehicle	6	Day 29	1.55 ± 0.25	0.13 ± 0.04††
250	6	Day 29	1.32 ± 0.25	0.15 ± 0.05
500	6	Day 29	1.03 ± 0.28	0.22 ± 0.04^
1,000	6	Day 29	1.48 ± 1.05	0.59 ± 0.23^

Note: Significance by two-sided Jonkheere-Terpstra trend test, †p < .001; significance by one-sided Jonkheere-Terpstra trend test, ††p < .001; significance versus vehicle by two-sided Dunn's test: *p < .01; significance versus vehicle by two-sided Dunn's test, **p < .001; significance by one-sided Dunnett's test, ^p < .01.
Before analyzing the experimental samples, positive and negative control samples (Rat MicroFlow®PLUS Kit) were analyzed.
Abbreviations: NR, not reported due to bone marrow toxicity insufficient number of RETs available for micronucleus analysis; *SD*, standard deviation.
[a]Only 1 animal was analyzed for %MN-RETs in the 1,000 mg/kg group due to bone marrow toxicity.

TABLE 8 Comet assay results in rats treated with acetaminophen in the 3-Day and 1-Month Studies

Treatment (mg·kg^{-1}·day^{-1})	Number of animals	Sampling time	Blood % tail intensity mean ± *SD*	Liver % tail intensity mean ± *SD*
3-Day Study				
Vehicle	5	Day 3	0.26 ± 0.08^{T}	0.18 ± 0.06^{TT}
500	5	Day 3	0.31 ± 0.11	0.22 ± 0.09
1,000	5	Day 3	0.41 ± 0.12*	0.27 ± 0.10
2,000	5	Day 3	0.34 ± 0.07	0.28 ± 0.07*
EMS[a], 200	3	Day 3	24.56 ± 1.94#	28.07 ± 1.64#
1-Month Study				
Vehicle	5	Day 29	0.17 ± 0.10	0.12 ± 0.09^{TTT}
250	5	Day 29	0.13 ± 0.08	0.20 ± 0.11
500	5	Day 29	0.19 ± 0.10	0.18 ± 0.06
1,000	5	Day 29	0.24 ± 0.15	0.34 ± 0.25**
EMS[a], 200	3	Day 29	26.41 ± 4.85#	30.79 ± 10.66#

Note: Significance by one-sided linear trend test, ^{T}p < .05; ^{TT}p < .01; ^{TTT}p < .001; significance versus vehicle by one-sided Dunnett's test,* p < .05; **p < .01; significance versus vehicle by one-sided t test, #p < .0001.
Mean and standard deviation (*SD*) of animal median values for % tail intensity per treatment group.
[a]EMS was used as positive control.

In the 1-Month Study, no biologically relevant and/or statistically significant increase in the %MN-RETs was observed in the acetaminophen treatment groups up to 500 mg·kg^{-1}·day^{-1} on Day 4. (Table 7) The 1,000 mg·kg^{-1}·day^{-1} treatment could not be evaluated for micronucleus inductions on Day 4 due to severe reduction in the %RETs. The micronucleus test conducted on Day 29 showed a statistically significant increase in the %MN-RETs at 1,000 mg·kg^{-1}·day^{-1}, which was considered biologically relevant as it exceeded the upper limit of the laboratory vehicle range (animal range 0.05–0.27%).

3.2.3 | Comet assay

In the 3-Day Study, treatment with acetaminophen resulted in a dose-related increase in DNA damage (% tail intensity) in peripheral

blood cells, which was statistically significant at 1,000 mg·kg^{-1}·day^{-1}. (Table 8) These increases were not considered biologically relevant as the % tail intensity was minimally increased (up to 1.6-fold at the 1,000 mg·kg^{-1}·day^{-1} treatment level) and did not exceed the upper limit of the laboratory historical vehicle range (group mean of animal median values range: 0.17–0.66%). In addition, there was no evidence of an increased formation of "clouds" indicative of cytotoxicity and/or severe genotoxicity when evaluating the peripheral blood cells of all animals. Treatment with acetaminophen also led to a dose-related increase in the % tail intensity in liver cells, which was statistically significant at 1,000 mg·kg^{-1}·day^{-1}. However, these increases were not considered biologically relevant as the % tail intensity was minimally increased (up to 1.6-fold at the 1,000 mg·kg^{-1}·day^{-1} treatment level) and did not exceed the upper limit of the laboratory vehicle range (group mean of animal median values range: 0.05–0.37%). There was no evidence of an increased formation of "clouds" indicative of cytotoxicity and/or severe genotoxicity when evaluating the liver cells of all animals, but the observed histopathological findings (i.e., single cell necrosis and chronic centrilobular inflammation) could have been of influence on the DNA damage response in the comet assay.

The 1-Month Study showed no biologically relevant and/or statistically significant increase in % tail intensity in peripheral blood cells in the acetaminophen treatment groups on Day 29. In liver cells, no biologically relevant and/or statistically significant increase in % tail intensity was noted up to 500 mg·kg^{-1}·day^{-1} on Day 29. At 1,000 mg·kg^{-1}·day^{-1}, 2 of 6 male rats showed small increases in % tail intensity from which 1 animal showed values slightly above the upper limit of the laboratory historical data range (animal median value range: 0.02–0.58%), while the % tail intensity in this dose group was within the laboratory historical data range (group mean of animal median values range: 0.05–0.37%). At histology, single cell and focal necrosis were observed in the liver of these rats. As it cannot be excluded that these histopathological findings were of influence on the DNA damage response, the small increases in the % tail intensity were not considered biologically relevant.

3.2.4 | Toxicokinetic results

Systemic exposure to acetaminophen following oral dosing of 1,000 mg·kg^{-1}·day^{-1} was assessed in the 1-Month Study. Mean plasma Cmax values of acetaminophen after a single (Day 1) and repeated (Day 28) oral dosing of 1,000 mg·kg^{-1}·day^{-1} were 153,600 ng/ml at Day 1 and 131,500 ng/ml at Day 28. Time to reach maximum plasma concentrations (Tmax [hr]) were 0.5 hr at Day 1 and 2–7 hr on Day 28. Peak plasma concentrations were generally reached at 0.5 hr and declined slowly between 0.5 and 24 hr after single dosing. After multiple treatment administrations, plasma concentrations remained constant between 0.5 and 7 hr and declined at 24 hr after dosing. Exposure after multiple dosing was comparable to single dosing.

4 | DISCUSSION

The *Pig-a* assay is a sensitive high throughput mutation detection assay and can be easily applied into standard in vivo toxicology testing since only peripheral blood is needed (Bryce *et al*., 2008; Miura *et al*., 2008; Schuler *et al*., 2011). The output of *Pig-a* analysis can be applied in helping to identify modes of actions of compounds. The need to validate the *Pig-a* assay drove the formation of the international multilaboratory *Pig-a* assay validation trial (Gollapudia *et al*., 2015). This trial was completed with the support of data on acetaminophen described in this paper (listed in Table 1 within the Gollapudia *et al*., 2015 publication) and the outcome of the trial was published by Gollapudia *et al*., 2015. The study data of the 41 compounds tested in the *Pig-a* assay in support of this trial are slowly being published and this paper describing the results of acetaminophen is one of them. A similar type of collaborative *Pig-a* validation study was conducted by the Mammalian Mutagenicity Study Group of the Japanese Environmental Mutagen Society and acetaminophen was also selected as one of the test compounds (Suzuki *et al*., 2016), albeit data were generated following only single oral treatment administration. It is important to note that a *Pig-a* Database has been established to make these data available to stakeholders (https://www.pharmacy.umaryland.edu/centers/cersi-files/, Shemansky et al. 2019).

Acetaminophen is an analgesic and antipyretic used to temporarily relieve pain and fever either alone or as an active ingredient in hundreds of over-the-counter and prescription medicines. Since acetaminophen was initially approved by the FDA in 1951 and was first marketed in the United States in 1953, it has been extensively evaluated for genotoxicity using both in vitro and in vivo assays with a variety of endpoints (IARC, 1990; NTP, 1993; Müller and Kasper, 1995; Bergman *et al*., 1996; Hantson *et al*., 1996; IARC, 1999; Matsushima *et al*., 1999; Oshida *et al*., 2008; Arun and Rabeeth, 2010; Salah *et al*., 2012). Regulatory and peer reviewed published data support that acetaminophen is nonmutagenic (as previously concluded from bacteria, in vitro mammalian cell, and in vivo transgenic rat assays) and noncarcinogenic (NTP, 1993; Bergman *et al*., 1996). The data suggest that acetaminophen has the potential to cause chromosomal damage at cytotoxic concentrations (Sasaki *et al*., 1983; Müller *et al*., 1991; Müller and Kasper, 1995; Bergman *et al*., 1996; Arun and Rabeeth, 2010; Salah *et al*., 2012). The potential for acetaminophen to cause chromosomal damage is observed following liver toxicity, which is a prominent observation in rats treated with greater than 500 mg·kg^{-1}·day^{-1} (Venkatesan *et al*., 2014). This profile in genotoxicity testing made acetaminophen an ideal addition to the 40 compounds selected for use in the international multilaboratory *Pig-a* assay validation trial (Gollapudia *et al*., 2015).

This body of work was undertaken to support the validation of the *Pig-a* assay through testing of the nonmutagenic compound acetaminophen.

4.1 | *Pig-a* endpoint

There were no statistically significant or dose-related increases in mutant phenotype RET (CD59-negative RETs) or RBC

(CD59-negative RBCs) frequencies (Figure 1) indicative of lack of a *Pig-a* gene mutation up to 1,000 mg·kg^{-1}·day^{-1} analyzed at multiple time points up to 29 days of dosing and following a 28-day recovery period. These findings are in line with the current literature (Suzuki *et al*., 2016) in which no changes in the *Pig-a* mutant frequency in peripheral blood of male Sprague Dawley rats were observed when treated with a single dose of acetaminophen by oral gavage at doses up to 2,000 mg·kg^{-1}·day^{-1} and evaluated pre-treatment and 1, 2 and 4 weeks post-treatment. Moreover, no changes in %RET were observed in any group related to the treatment.

This current body of work agrees with the historic literature that acetaminophen is not mutagenic (Bergman *et al*., 1996; Kanki *et al*., 2005; Matsushita *et al*., 2013). Peripheral RBCs in rats have a lifespan of approximately 90 days, the population of erythroid cells is slowly replaced by the bone marrow. If *Pig-a* mutations are induced, then the *Pig-a* mutant cells will gradually accumulate in the peripheral RBCs over time and thus in longer duration studies, the later study blood samples should be able to detect increases in mutant phenotype RBC (CD59-negative RBCs) frequencies. However, in the longer duration study (1-Month Study) of this presented body of work, the last sampling time point (Day 57) showed no increases in *Pig-a* mutant frequency, which supports the lack of mutagenicity of acetaminophen.

Acetaminophen treatments of ≥1,000 mg·kg^{-1}·day^{-1} inhibited RBC proliferation with a dose-related decrease in the %RETs to less than 10% of the vehicle values in the range finding assay. However, in the 3-Day Study temporary increases in %RETs were observed on Day 15 at 1,000 and 2,000 mg·kg^{-1}·day^{-1} of the *Pig-a* assessment. (Figure 1) Stimulated erythropoiesis likely explains this finding as there was a marked reduction in the %RETs on Day 4 of the micronucleus assessment. Also supporting this conclusion is the observed decrease in RBCs in the 1-Month Study on Days 8, 16, and 29 at 1,000 mg·kg^{-1}·day^{-1}. Please note that the %RETs were measured within both the *Pig-a* and micronucleus endpoints but by different labeling techniques. Therefore, while values were very close in most cases, they were not identical. Other hematological parameters were impacted at doses ≥1,000 mg·kg^{-1}·day^{-1} such as with the 1-Month Study there were decreases in %RETs, neutrophils, lymphocytes, monocytes, and basophils. By Day 8 most of the hematology parameters in the 1-Month Study had decreased values except the MCV and MCH; however, most parameters rebounded by Day 16 except the RBCs, %RETs, MCV, and MCH (Appendix 1). The data demonstrate that the *Pig-a* endpoint was negative in peripheral blood RETs and RBCs from acetaminophen-(≤1,000 mg·kg^{-1}·day^{-1}) treated rats dosed up to 29 days and that temporarily marked reduction in the %RET did not trigger a positive response in the *Pig-a* assay. The changes in the hematopoiesis did not appear to cause a positive finding in the *Pig-a* endpoint which was also demonstrated by the work of Kenyon *et al*., 2015 and Nicolette *et al*., 2018.

Exposure data demonstrated high acetaminophen mean plasma Cmax values after single (Day 1) and repeated (Day 28) oral dosing of 1,000 mg·kg^{-1}·day^{-1} (153,600 and 131,500 ng/ml, respectively). These exposure data support the presence of high levels of acetaminophen to the target cells hence confirming the robustness of the negative results in the *Pig-a* endpoint of this study.

For all *Pig-a* studies under this body of work (except the 2-Week Study), a deselection of animals with high spontaneous *Pig-a* mutant frequencies prior to study start was conducted. Although the percentage of animals with high spontaneous *Pig-a* mutant frequencies was low (~1%), deselection increased the robustness of the *Pig-a* assay. Deselection of animals with high spontaneous *Pig-a* mutant frequencies is considered good practice to increase the sensitivity of the assay (Gollapudia *et al*., 2015).

4.2 | Hematology

Hematology observations following acetaminophen treatments of ≥500 mg·kg^{-1}·day^{-1} showed a statistically significant and dose–response reduction in WBCs, neutrophils, and lymphocytes after 14 days of dosing as well as several RBC parameters (RBC and RET counts; hemoglobin, MCHC, MCH and MCV levels) mainly at 1,000 mg·kg^{-1}·day^{-1} after 28 days of dosing. These RBC findings were all slight, transient, and values were comparable to vehicle treated animals toward the end of the dosing period, suggesting an adaptive response. These changes in hematology parameters could be attributed to the toxicities observed in the hematopoietic cells used in the *Pig-a*, micronucleus, and comet endpoints.

4.3 | Micronucleus endpoint

Acetaminophen ≥1,000 mg·kg^{-1}·day^{-1} led to marked reduction in %RETs and no increases in the %MN-RETs were observed with any treatment in the 3-Day Study. (Table 7) The 2,000 mg·kg^{-1}·day^{-1} acetaminophen treatment could not be evaluated for micronuclei due to the reduction in the %RETs. No biologically relevant and/or statistically significant increase in %MN-RETs was observed in the acetaminophen-treated rats in the 1-Month Study with Day 4 samples. However, the top treatment level (1,000 mg·kg^{-1}·day^{-1}) samples could not be evaluated for micronucleus induction due to a severe reduction in %RETs. The %RETs at 1,000 mg·kg^{-1}·day^{-1} in the 1-Month Study rebounded back to the background level as observed in the Day 29 samples; however, this effect was mirrored by a small, biologically relevant, and statistically significant increase in the %MN-RETs. This increase was may be due to stimulated erythropoiesis. The small increase in the %MN-RETs should be carefully interpreted given that it is not uncommon to see micronucleus induction at treatment levels which impact erythropoiesis. Changes in erythropoiesis can cause nongenotoxic-induced findings in the in vivo micronucleus endpoint (Tweats, *et al*., 2007; ICH, 2011). Baumeister *et al*. (1994), also observed increased micronucleus frequencies in rats treated at high doses of acetaminophen inducing marked liver and bone marrow toxicity (Baumeister *et al*., 1994). Based on hematotoxicity observed at this does, we concluded that the micronucleus endpoint was negative in peripheral blood RETs from acetaminophen-(≤ 1,000 mg·kg^{-1}·day^{-1}) treated rats up to 29 days.

4.4 | Comet endpoint

Following 3 days or 1 month of daily treatment with acetaminophen, peripheral blood cells, and liver cells had no biologically relevant increases in DNA damage (% tail intensity). (Table 8) Upon histopathologic evaluation, single cell and focal necrosis were observed in the liver of these rats. As it cannot be excluded that these histopathological findings influenced the DNA damage response, the small increases in DNA damage observed in 2 of 6 male rats on Day 29 at 1,000 mg·kg^{-1}·day^{-1} were not considered biologically relevant. Per the OECD TG-489, histopathological changes are considered a relevant measure of tissue toxicity for use in the comet endpoint (OECD, 2016b). Acetaminophen treatment levels of 500 mg·kg^{-1}·day^{-1} and, to a greater extent, 1,000 mg·kg^{-1}·day^{-1} caused histopathological findings in the centrilobular region of the liver characterized by focal/single cell necrosis, inflammation, focal/multifocal fibrosis (with some bridging), brown pigmented macrophages, hepatocellular vacuolization, and/or intravenous cell debris in centrilobular veins. These findings confirm that at an acetaminophen treatment level of ≥500 mg·kg^{-1}·day^{-1} for ≥3 days there is some histopathological impact to the rat liver. Clinical chemistry changes can also help validate tissue toxicity following dosing (OECD, 2016b). In these studies, there were treatment related changes in some clinical chemistry parameters including bilirubin, ALT, GLDH, total bile acids, and calcium at ≥500 mg·kg^{-1}·day^{-1} and statistically significant findings at 1,000 mg·kg^{-1}·day^{-1} with dosing ≥14 days. These histopathology and clinical chemistry biomarkers support the conclusion that these high treatment levels of acetaminophen appear to have caused liver toxicity. Data from El Morsy and Kamel, 2015 support our observations (El Morsy and Kamel, 2015). Rats receiving a single treatment of acetaminophen at 2,000 mg.kg^{-1} showed marked liver damage, gross necrotic areas, as well as increased serum aminotransferases with accompanying increases in % DNA migration at 24 hr after dosing. Based on this information, we concluded that the comet endpoint was negative in both peripheral blood cells and liver cells from acetaminophen-(≤1,000 mg·kg^{-1}·day^{-1}) treated rats treated up to 29 days.

5 | CONCLUSION

Acetaminophen when administered at dose levels of 250, 500, 1,000, or 2,000 mg·kg^{-1}·day^{-1} to male rats in a series of 3 studies, each with progressively longer durations up to 29 days and recovery periods up to 28 days, did not induce any biologically relevant increases in mutant phenotype (CD59-negative) erythrocytes indicative of a *Pig-a* gene mutation. Likewise, no biologically relevant increases in the level of DNA damage in peripheral blood cells or liver cells via comet assessments nor in the percentage of micronucleated RETs were observed except in the high dose group of 1,000 mg·kg^{-1}·day^{-1} at hematotoxic exposures. At 1,000 mg·kg^{-1}·day^{-1}, Cmax values of acetaminophen on Day 28 were 153,600 ng/ml and 131,500 ng/ml after single and repeat dosing, respectively, which were multiples over that of clinical therapeutic exposures (2.1 to 6.1 fold for single doses of 4,000 mg and 1,000 mg, respectively, and 11.5 fold for multiple dose of 4,000 mg) (FDA, 2002). The changes observed in the histopathology, hematology, and in %RETs in the *Pig-a* and micronucleus endpoints indicate that the high levels of acetaminophen used in this study induced toxicity to the target cells; these data support the aggressive efforts put forth in this body of work to uncover any acetaminophen genotoxicity.

Based on the lack of responses for the *Pig-a*, micronucleus, and comet endpoints at treatment levels inducing toxicity, this block of studies supports that acetaminophen is not genotoxic under these experimental conditions. Nonetheless a small, biologically relevant, and statistically significant increase in the %MN-RETs was observed at 1,000 mg·kg^{-1}·day^{-1} in the 1-Month Study. However, this observation was preceded by a severe reduction in %RETs which rebounded back to the background level. This increase was maybe due to stimulated erythropoiesis. The small increase in the %MN-RETs should be carefully interpreted given that it is not uncommon to see micronucleus induction at treatment levels which impact erythropoiesis. Changes in erythropoiesis can cause nongenotoxic induced findings in the in vivo micronucleus endpoint (Tweats *et al*., 2007; ICH, 2011).

The data generated were reproducible across this series of studies conducted, of high quality by inclusion of various toxicity parameters, and in line with genotoxicity findings from current literature for acetaminophen. Hence, these data are valid for contribution to the international multilaboratory *Pig-a* assay validation trial.

ACKNOWLEDGMENTS

The authors thank all colleagues from Rodent Toxicology, Autopsy and Histoprocessing groups of Janssen Research and Development, Beerse, Belgium for their excellent technical assistance. The authors acknowledge Kathleen Boyle, PhD, from 4 Learning Group, LLC (Exton, PA) who, under the direction of the authors, supported manuscript preparation and managed the review process. They also thank Litron Laboratories for their advice in setting up the *Pig-a* assay and for providing the prototype Rat MutaFlow® kits. The Janssen Pharmaceutical Companies of Johnson & Johnson provided funding for the studies. Johnson & Johnson Consumer Inc, McNeil Consumer Healthcare Division provided for support by an independent medical writer.

CONFLICT OF INTEREST

All authors were employees of The Janssen Pharmaceutical Companies of Johnson & Johnson. All authors had full access to study data and had final responsibility to submit the manuscript for publication.

AUTHOR CONTRIBUTIONS

B.-j.V.D.L., T.V.D., and A.S. designed the *Pig-a* experiment protocol/study design, obtained requisite animal use approvals, and supervised the laboratory work. B.-j.V.D.L. and T.V.D. designed the micronucleus experiments, obtained requisite animal use approvals, and supervised the laboratory work. B.-j.V.D.L., K.D.V., M.V.H., and S.D.J. designed comet experiments, obtained requisite animal use approvals, and supervised the laboratory work. B.-j.V.D.L., S.W., T.V.D., K.D.V., A.S., F.T., H.G., M.V.H., and S.D.J. conducted data review and analyses. B.-j.V.D.L., S.W., F.T., H.G., M.V.H., S.D.J., and J.V.G. drafted and critically

reviewed and revised the manuscript. B.-j.V.D.L., S.W., T.V.D., K.D.V., A.S., F.T., H.G., M.V.H., S.D.J., and J.V.G. provided final approval of the version to be published and agree to be accountable for all aspects of work and for appropriately investigating and resolving questions.

DATA AVAILABILITY STATEMENT

The data sharing policy of Janssen Pharmaceutical Companies of Johnson & Johnson is available at https://www.janssen.com/clinical-trials/transparency. As noted on this site, requests for access to the study data can be submitted through Yale Open Data Access (YODA) Project site at http://yoda.yale.edu.

REFERENCES

Abdel-Zaher, A.O., Abdel-Hady, R.H., Mahmoud, M.M. and Farrag, M.M. (2008) The potential protective role of alpha-lipoic acid against acetaminophen-induced hepatic and renal damage. *Toxicology*, 243, 261–270.

Aidoo, A., Morris, S.M. and Casciano, D.A. (1997) Development and utilization of the rat lymphocyte *hprt* mutation assay. *Mutation Research*, 387, 69–88.

Arun, T. and Rabeeth, M. (2010) Genotoxic effect of paracetamol containing tablets in cultured human lymphocytes. *International Journal of Biomedical Research*, 1(2), 21–30.

Avlasevich, S.L., Labash, C., Torous, D.K., Bemis, J.C., MacGregor, J.T. and Dertinger, S.D. (2018) In vivo pig-a and micronucleus study of the prototypical aneugen vinblastine sulfate. *Environmental and Molecular Mutagenesis*, 59(1), 30–37.

AVMA Panel on Euthanasia. (2001) 2000 report of the American Veterinary Medical Association (AVMA) panel on euthanasia. *JAVMA*, 218(5), 669–696.

Baumeister, M., Miller, B., Gocke, E., Krauser, K., Speit, G., Sagelsdorff, P., Arni, P. and Thomas, H. (1994) Paracetamol-induced chromosomal damage under in vivo conditions: rationale for a threshold concept and its clinical relevance. *Toxicology Letters*, 74(Supplement 1), 7.

Belgian Law. 1991. *Belgian Law (October 18, 1991): Protection of vertebrate animals used for experimental and other scientific purposes. Royal Decree of November 14, 1993 for the protection of laboratory animals*. Brussels, Belgium.

Bergman, K., Müller, L. and Teigen, S.W. (1996) The genotoxicity and carcinogenicity of paracetamol: a regulatory review. *Mutation Research*, 349, 263–288.

Boerrigter, M.E., Dolle, M.E., Martus, H.J., Gossen, J.A. and Vijg, J. (1995) Plasmid-based transgenic model for studying in vivo mutations. *Nature*, 377, 657–659.

Boyd, E.M. and Bereczky, G.M. (1966) Liver necrosis from paracetamol. *British Journal of Pharmacology and Chemotherapy*, 26, 606–614.

Bright, J., Aylott, M., Bate, S., Geys, H., Jarvis, P., Saul, J. and Vonk, R. (2011) Recommendations on the statistical analysis of the comet assay. *Pharmaceutical Statistics*, 10, 485–493.

Bryce, S.M., Bemis, J.C. and Dertinger, S.D. (2008) In vivo mutation assay based on the endogenous pig-a locus. *Environmental and Molecular Mutagenesis*, 49, 256–264.

Chatterjee, S. and Mayor, S. (2001) The GPI-anchor and protein sorting. *Cellular and Molecular Life Sciences*, 58, 1969–1987.

Council Directive. 2007. European Convention (ETS no. 123) for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. Council Directive of November 24, 1986 (86/609/EEG).

Dertinger, S.D. and Heflich, R.H. (2011) In vivo assessment of pig-a gene mutation recent developments and assay validation. *Environmental and Molecular Mutagenesis*, 52, 681–684.

Dertinger, S.D., Phonethepswath, S., Weller, P., Nicolette, J., Murray, J., Sonders, P., Vohr, H.W., Shi, J., Krsmanovic, L., Gleason, C., Custer, L., Henwood, A., Sweder, K., Stankowski, L.F., Jr., Roberts, D.J., Giddings, A., Kenny, J., Lynch, A.M., Defrain, C., Nesslany, F., van der Leede, B.J., Van Doninck, T., Schuermans, A., Tanaka, K., Hiwata, Y., Tajima, O., Wilde, E., Elhajouji, A., Gunther, W.C., Thiffeault, C.J., Shutsky, T.J., Fiedler, R.D., Kimoto, T., Bhalli, J.A., Heflich, R.H. and MacGregor, J.T. (2011) International pig-a gene mutation assay trial: evaluation of transferability across 14 laboratories. *Environmental and Molecular Mutagenesis*, 52(9), 690–698.

Dertinger, S.D., Phonethepswath, S., Avlasevich, S.L., Torous, D.K., Mereness, J., Bryce, S.M., Bemis, J.C., Bell, S., Weller, P. and MacGregor, J.T. (2012) Efficient monitoring of in vivo pig-a gene mutation and chromosomal damage: summary of 7 published studies and results from 11 new reference compounds. *Toxicological Sciences*, 130, 328–348.

Dobrovolsky, V.N., Miura, D., Heflich, R.H. and Dertinger, S.D. (2010) The in vivo pig-a gene mutation assay, a potential tool for regulatory safety assessment. *Environmental and Molecular Mutagenesis*, 51, 825–835.

Dunn, O.J. (1964) Multiple comparisons using rank sums. *Technometrics*, 6 (3), 241–252.

El Morsy, E.M. and Kamel, R. (2015) Protective effect of artichoke leaf extract against paracetamol-induced hepatotoxicity in rats. *Pharmaceutical Biology*, 53(2), 167–173.

Ferguson, M.A.J., Hart, G.W. and Kinoshita, T. (2017) Glycosylphosphatidylinositol anchors. In: Varki, A., Cummings, R.D., Esko, J.D., et al. (Eds.) *Essentials of Glycobiology [internet]*, Vol. Chapter 12, 3rd edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 2015–2017. https://doi.org/10.1101/glycobiology.3e.012. Available at: https://www.ncbi.nlm.nih.gov/books/NBK453072/.

Food and Drug Administration: *McNeil Consumer & Specialty Pharmaceuticals Briefing Information for the September 19*, 2002 *Meeting of the Nonprescription Drugs Advisory Committee*. Available at: https://wayback.archive-it.org/7993/20170405154834/https://www.fda.gov/ohrms/dockets/ac/02/briefing/3882B1_13_McNeil-Acetaminophen.pdf [Accessed 21st January 2020].

Gollapudia, B.B., Lynch, A.M., Heflich, R.H., Dertinger, S.D., Dobrovolsky, V.N., Froetschl, R., Horibata, K., Kenyon, M.O., Kimoto, T., Lovell, D.P., Stankowski, L.F., Jr., White, P.A., Witt, K.L. and Tanir, J.Y. (2015) The in vivo pig-a assay: a report of the international workshop on genotoxicity testing (IWGT) workgroup. *Mutation Research*, 783, 23–35.

Gossen, J.A., de Leeuw, W.J.F., Tan, C.H.T., Lohman, P.H.M., Berends, F., Knook, D.L., Zwarthoff, E.C. and Vijg, J. (1989) Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying mutations in vivo. *Proceedings of the National Academy of Sciences USA*, 86, 7981–7985.

Hantson, P., de Saint-Georges, L., Mahieu, P., Léonard, E.D., Crutzen-Fayt, M.C. and Léonard, A. (1996) Evaluation of the ability of paracetamol to produce chromosome aberrations in man. *Mutation Research*, 368, 293–300.

Heddle, J.A., Shaver-Walker, P., Tao, K.S. and Zhang, X.B. (1995) Treatment protocols for transgenic mutation assays in vivo. *Mutagen*, 10, 467–470.

IARC. 1990. Paracetamol (acetaminophen). Pharmaceutical drugs. (IARC monographs on the evaluation of carcinogenic risks to humans volume 50). Available at: http://publications.iarc.fr/68.

IARC. 1999. Paracetamol. Some Chemicals that Cause Tumours of the Kidney or Urinary Bladder in Rodents and Some Other Substances. (IARC monographs on the evaluation of carcinogenic risks to humans). Available at: https://publications.iarc.fr/91.

ICH. 2011. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). *ICH Harmonised Tripartite Guideline S2(R1) Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use*, S2(R1), Current *Step 4* version, dated November 9, 2011.

ICH. 2017. International Conference on Harmonisation of Technical Requirmens for Registration of Pharmaceutical for Human Use (ICH).

ICH M7(R1) guidance on assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk required testing: International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use.

Jonckheere, A.R. (1954) A distribution-free k-sample test against ordered alternatives. *Biometrika*, 41, 133–145.

Kanki, K., Nishikawa, A., Masumura, K., Umemura, T., Imazawa, T., Kitamura, Y., Nohmi, T. and Hirose, M. (2005) In vivo mutational analysis of liver DNA in *gpt* delta transgenic rats treated with the hepatocarcinogens N-nitrosopyrrolidine2-amino-3-methylimidazo [4,5-f] quinoline, and di (2-ethylhexyl) phthalate. *Molecular Carcinogenesis*, 42, 9–17.

Kenyon, M.O., Coffing, S.L., Ackerman, J.I., Gunther, W.C., Dertinger, S.D., Criswell, K. and Dobo, K.L. (2015) Compensatory erythropoiesis has no impact on the outcome of the in vivo pig-a mutation assay in rats following treatment with the haemolytic agent 2-butoxyethanol. *Mutagenesis*, 30(3), 325–334.

Kohler, S.W., Provost, G.S., Fieck, A., Kretz, P.L., Bullock, W.O., Putman, D.L., Sorge, J.A. and Short, J.M. (1991) Spectra of spontaneous and induced mutations using a lambda ZAPRlacI shuttle vector in transgenic mice. *Proceedings of the National Academy of Sciences USA*, 88, 7958–7962.

Levene, H. (1960) Contributions to probability and statistics: essays in honor of Harold Hotelling. In: Olkin, I., et al. (Eds.). Stanford University Press, pp. 278–292.

Manjanatha, M.G., Shelton, S.D., Aidoo, A., Lyn-Cook, L.E. and Casciano, D. A. (1998) Comparison of in vivo mutagenesis in the endogenous *Hprt* gene and the *lacI* transgene in big blue(R) rats treated with 7,12-dimethylbenz[a]anthracene. *Mutation Research*, 401, 165–178.

Matsushima, T., Hayashi, M., Matsuoka, A., Ishidate, M., Jr., Miura, K.F., Shimizu, H., Suzuki, Y., Morimoto, K., Ogura, H., Mure, K., Koshi, K. and Sofuni, T. (1999) Validation study of the in vitro micronucleus test in a Chinese hamster lung cell line (CHL/IU). *Mutagenesis*, 14(6), 569–580.

Matsushita, K., Kijima, A., Ishii, Y., Takasu, S., Jin, M., Kuroda, K., Kawaguchi, H., Miyoshi, N., Nohmi, T., Ogawa, K. and Umemura, T. (2013) Development of a medium-term animal model using *gpt* Delta rats to evaluate chemical carcinogenicity and genotoxicity. *Journal of Toxicologic Pathology*, 26, 19–27.

Miura, D., Dobrovolsky, V.N., Kasahara, Y., Katsuura, Y. and Heflich, R.H. (2008) Development of an in vivo gene mutation assay using the endogenous pig-a gene: I. flow cytometric detection of CD59-negative peripheral red blood cells and CD48-negative spleen T-cells from the rat. *Environmental and Molecular Mutagenesis*, 49, 614–621.

Müller, L. and Kasper, P. (1995) OTC pharmaceuticals and genotoxicity testing: The paracetamol, anthraquinone, and griseofulvin cases. *Archives of Toxicology Supplement*, 17, 312–325.

Müller, L., Kasper, P. and Madle, S. (1991) Further investigations on the clastogenicity of paracetamol and acetylsalicylic acid in vitro. *Mutation Research*, 263, 83–92.

National Toxicology Program (NTP). (1993) *Technical Report Series No. 394, Toxicology and carcinogenesis studies of acetaminophen (CAS NO. 103–90-2) in F344/N rats and B6C3Fl mice (feed studies)*. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health. Available at: https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr394.pdf.

Nicolette, J., Murray, J., Sonders, P., Leroy, B. (2018) A regenerative erythropoietic response does not increase the frequency of Pig-a mutant reticulocytes and erythrocytes in Sprague-Dawleyrats. *Environmental and Molecular Mutagenesis*, 59(1), 91–95.

Organization for Economic Co-operation and Development (OECD). 2016a. Guidelines for testing of chemicals, section 4 health effects. Test no 489: in vivo mammalian alkaline comet, adopted by the OECD council by written procedure on July 29, 2016 [C(2016)103]. Available at: https://www.oecd.org/env/test-no-489-in-vivo-mammalian-alkaline-comet-assay-9789264264885-en.htm.

OECD. 2016b. Guidelines for testing of chemicals, section 4 health effects. Test no. 474: Mammalian erythrocyte micronucleus test, adopted by the OECD council by written procedure on July 29, 2016 [C(2016)103]. Available at: https://www.oecd.org/env/test-no-474-mammalian-erythrocyte-micronucleus-test-9789264264762-en.htm.

Oshida, K., Iwanaga, E., Miyamoto-Kuramitsu, K. and Miyamoto, Y. (2008) An in vivo comet assay of multiple organs (liver, kidney and bone marrow) in mice treated with methyl methanesulfonate and acetaminophen accompanied by hematology and/or blood chemistry. *Journal of Toxicological Sciences*, 33(5), 515–524.

Registration, Evaluation, Authorization and Restriction of Chemicals (REACH). 2006. *Regulation (EC) No 1907/2006 of the European Parliament and of the Council of December 18, 2006*.

Salah, S.H., Abdouh, S., Boolesand, H. and Abdel Rahim, E.A. (2012) Effect of *Zingiber officinale* on paracetamol-induced genotoxicity in male rats. *Journal of Medicinal Plants Research*, 6(41), 5425–5434.

Sasaki, M., Yoshida, S. and Hiraga, K. (1983) Additional effect of acetaminophen on the mutagenicity and clastogenicity of N-methyl-N-nitro-N-nitrosoguanidine in cultured Chinese hamster CHO-kl cells. *Mutation Research*, 122, 367–372.

Schuler, M., Gollapudi, B.B., Thybaud, V. and Kim, J.H. (2011) Need and potential value of the pig-a in vivo mutation assay—A HESI perspective. *Environmental and Molecular Mutagenesis*, 52, 685–689.

Shapiro, S.S. and Wilk, M.B. (1964) An analysis of variance test for normality (complete samples). *Biometrika*, 52(3), 591–611.

Shemansky, J.M., McDaniel, L.P., Klimas, C., Dertinger, S.D., Dobrovolsky, V. N., Kimoto, T., Horibata, K., Polli, J.E., Heflich, R.H. (2019) Pig-a gene mutation database. *Environmental Molecular Mutagenesis*. 60(8):759–762.

Strauss, G.H. and Albertini, R.J. (1979) Enumeration of 6-thioguanine-resistant peripheral blood lymphocytes in man as a potential test for somatic mutations arising in vivo. *Mutation Research*, 61, 353–379.

Suzuki, Y., Goto, K., Nakayama, Y., Saratani, M., Takata, T., Okamoto, T. and Okazaki, S. (2016) Evaluation of a single-dose PIGRET assay for acetaminophen in rats compared with the RBC pig-a assay. *Mutation Research*, 811, 16–20.

Tweats, D.J., Blakey, D., Heflich, R.H., Jacobs, A., Jacobsen, S.D., Morita, T., Nohmi, T., O'Donovan, M.R., Sasaki, Y.F., Sofuni, T., Tice, R. and IWGT Working Group. (2007) Report of the IWGT working group on strategies and interpretation of regulatory in vivo tests I. Increases in micronucleated bone marrow cells in rodents that do not indicate genotoxic hazards. *Mutation Research*, 627(1), 78–91.

Venkatesan, P.S., Deecaraman, M., Vijayalakshmi, M. and Sakthivelan, S.M. (2014) Sub-acute toxicity studies of acetaminophen in Sprague Dawley rats. *Biological and Pharmaceutical Bulletin*, 37(7), 1184–1190.

Verbeke, G. and Molenberghs, G. (2000) *Linear mixed models for longitudinal data*. New York: Springer-Verlag.

Von Tungeln, L.S., Williams, L.D., Doerge, D.R., Shaddock, J.G., McGarrity, L.J., Morris, S.M., Mittelstaedt, R.A., Heflich, R.H. and Beland, F.A. (2007) Transplacental drug transfer and frequency of *Tk* and *Hprt* lymphocyte mutants and peripheral blood micronuclei in mice treated transplacentally with zidovudine and lamivudine. *Environmental and Molecular Mutagenesis*, 48, 258–269.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: van der Leede B-j, Weiner S, Van Doninck T, et al. Testing of acetaminophen in support of the international multilaboratory in vivo rat *Pig-a* assay validation trial. *Environ Mol Mutagen*. 2020;61:508–525. https://doi.org/10.1002/em.22368