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

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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

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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 β-thioguanine) and the transgenes-based assays: Iac2 and lacI (Gossen et al., 1989; Kohler et al., 1991; Boerrigter et al., 1995; Hedde 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 Hellich, 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 Specpre, 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, ethylene-diaminetetraacetic acid disodium salt dihydrate (EDTA- Na2·2H2O), 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
| Type of study                      | Purpose/Endpoints                      | Treatment<sup>a</sup> (mg kg<sup>−1</sup> day<sup>−1</sup>) | N per group | Dosing days | Observation days | Bleeding days | Tissue sampling days | Day of terminal kill |
|-----------------------------------|----------------------------------------|-------------------------------------------------------------|-------------|-------------|------------------|---------------|--------------------|---------------------|
| 3-Day Study                       |                                        |                                                              |             |             |                  |               |                    |                     |
| Dose Range Finding Study<sup>b</sup> | toxicity                           | 250, 500, 1,000, 2,000                                      | 3           | 1-3         |                  | NA            | NA                 | 4                   |
|                                   | Mortality and clinical observations   | All                                                          |             |             | Daily: 1-4       |               |                    |                     |
|                                   |                                        |                                                              |             |             | 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, 2, 3: before dosing; Day 4: before terminal kill |               |                    |                     |
|                                   |                                        |                                                              |             |             |                  |               |                    |                     |
| 3-Day Study                       | Pig-α<sup>c</sup> and micronucleus (MN)<sup>d</sup> assays | Vehicle 500, 1,000, 2,000                                    | 6           | 1-3         |                  | MN: 4          | NA                 | 45                  |
|                                   |                                       | ENU (40)                                                     | 3           | 1-3         |                  | Pig-α: 4, 15, 30, 45 | NA                 | 45                  |
|                                   |                                       | Vehicle 500, 1,000, 2,000                                    | 5           | 1-3         |                  | Comet: 3       | Comet: 3            | 3                   |
|                                   |                                        | ENU (40)                                                     |             |             |                  |                | Histopathology: 3  |                     |
|                                   |                                        | Vehicle, 500, 1,000, 2,000                                   |             |             |                  | Comet: 3       | Comet: 3            | 3                   |
|                                   |                                        | ENU (40)                                                     |             |             |                  |                | Histopathology: 3  |                     |
|                                   |                                        | Vehicle, 500, 1,000, 2,000                                   |             |             |                  |                |                    |                     |
|                                   | Mortality and clinical observations   | All                                                          |             |             | Daily: Days 1-45  |               |                    |                     |
|                                   |                                        |                                                              |             |             | Time-related: Day 1 at 0, 1, 3, 5 hr; Days 2 and 3 at 0, 1, 3 hr |               |                    |                     |
|                                   | Body weight                           | Vehicle, 500, 1,000, 2,000                                   |             |             |                  | Days 1, 2, 3 before dosing |               |                    |
|                                   |                                        | ENU (40)                                                     |             |             |                  | Days 8, 15, 22, 29, 36, 43, 46 |               |                    |

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

<sup>a</sup>Vehicle, Acetaminophen (250, 500, 1,000 or 2,000), ENU (40) or EMS (200).

<sup>b</sup>The Dose Range Finding Study was conducted prior to initiation of the Definitive Study.

<sup>c</sup>Pig-α assay: peripheral blood.

<sup>d</sup>Micronucleus (MN) assay: peripheral blood.

<sup>e</sup>Comet assay: liver and peripheral blood.

<sup>f</sup>Histopathology: liver.


### 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 kg\(^{-1}\) 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 kg\(^{-1}\) 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 kg\(^{-1}\) 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, 250, 1,000 or 2,000 mg kg\(^{-1}\) day\(^{-1}\) of acetaminophen for 3 consecutive days. A separate group of 3 animals was treated with 40 mg kg\(^{-1}\) 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 kg\(^{-1}\) 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 kg\(^{-1}\) 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 kg\(^{-1}\) day\(^{-1}\) of acetaminophen for 15 consecutive days;
| Type of study        | Purpose/endpoint                                                                 | Treatment$^a$  | N per group | Dosing days | Observation days | Bleeding days | Tissue sampling days | Day of terminal kill |
|---------------------|---------------------------------------------------------------------------------|----------------|-------------|-------------|------------------|--------------|--------------------|---------------------|
| 1-Month Study       | Genetic Toxicity                                                                | Vehicle        | 6           | 1–29        |                  |              |                    |                     |
|                     | Pig-a$^b$, MN$^c$ and comet assays$^d$, hematology, clinical chemistry and histopathology$^e$ | 250, 500, 1,000 |             |             |                  |              |                    |                     |
|                     | Pig-a assay$^b$                                                                  | ENU (40)       | 3           | 1–3         |                  |              |                    |                     |
|                     | Comet assay$^c$ and histopathology$^d$                                           | EMS (200)      | 3           | 28–29       |                  |              |                    |                     |
|                     | 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$^b$ and histopathology$^e$ | Vehicle 1,000 | 6           | 1–29           | Pig-a: −1, 15, 29 | 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, 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)          |
|                     | Mortality and clinical observations                                               | All            |             |             | Daily: 1–29    |              |                    |                     |
|                     |                                                                                   |                |             |             | Time-related: Days 1, 8, 15, 22, 28 at |              |                    |                     |
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 (i.e., 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 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, non-overlapping 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 1900g. 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 Bonferroni–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 10⁶ and RET percent (%RET) as described in Avalasevich et al. (Avalasevich et al., 2018). Accordingly, CD59-negative RBC and CD59-negative RET per 10⁶ 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·kg⁻¹·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: †p < .05; ††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 pretreatment 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 erythropagocytosis, 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.
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 kg\(^{-1}\) 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 kg\(^{-1}\) 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 16, 30, and 45).

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

| Treatment (mg kg\(^{-1}\) 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       | 977.7 ± 72\(^8\)                              | 414.2 ± 65\(^8\)                              |
| 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       | 324.7 ± 78.4                                  | 40.3 ± 98.3                                   |
| ENU\(^a\), 40                        | 3                 | 3.3 ± 0.6       | 256.7 ± 62.0\(^8\)                            | 282.7 ± 77.4\(^8\)                            |
| Day 45                               |                   |                 |                                               |                                               |
| Vehicle                              | 6                 | 3.1 ± 0.8\(^{TT}\) | 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\(^*\) | 385.7 ± 92.8                                  | 15.1 ± 36.2                                   |
| ENU\(^a\), 40                        | 3                 | 2.7 ± 0.7       | 382.7 ± 26.3\(^8\)                            | 305.9 ± 59.5\(^8\)                            |

Note: Significance versus vehicle by two-sided Dunnett’s test: \(^*\)\(p < .05\); \(^{*\*}\)\(p < .001\); significance by two-sided linear trend test: \(^{TT}\)\(p < .05\); \(^{TT\*}\)\(p < .001\); significance versus vehicle by one-sided t test: \(^{T}\)\(p < .05\); \(^{TT}\)\(p < .001\); significance versus vehicle by one-sided t test: \(^{T}\)\(p < .05\).

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 kg\(^{-1}\) 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.
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 kg⁻¹ day⁻¹ resulted in slight decreases in %RETs on Day 29 but by Day 57, the %RET had recovered (only 1,000 mg kg⁻¹ 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 kg⁻¹ 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 kg⁻¹ day⁻¹ treatment could not be evaluated for micronucleus induction due to severe reduction in the %RET. The loss of the data from the peripheral blood resulted in the conclusions of this study as being an exception to the OECD 474 guidance (OECD, 2016a).

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

| Treatment (mg kg⁻¹ day⁻¹) | Number of animals | %RETs mean ± SD | Number of CD59-Neg RBCs per 10⁶ RBCs mean ± SD | Number of CD59-Neg RETs per 10⁶ 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                                     |
| ENUa, 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                                     |
| ENUa, 40                  | 3                 | 3.1 ± 0.4       | 79.5 ± 12.3#                                  | 356.2 ± 64.4#                                 |
| Day 29                    |                   |                 |                                               |                                               |
| Vehicle                   | 12                | 3.0 ± 0.5†      | 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                                     |
| ENUa, 40                  | 3                 | 3.7 ± 0.7       | 211.0 ± 26.9#                                 | 291.4 ± 33.7#                                 |
| Day 57b                   |                   |                 |                                               |                                               |
| 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: †p < .05; significance versus vehicle by one-sided t test: #p < .001.

Abbreviations: RBCs, red blood cells; RETs, reticulocytes; SD, standard deviation.

aENU was used as positive control.

bOnly Vehicle and 1,000 mg kg⁻¹ day⁻¹ were included in this Day 57 recovery group and hence available for Pig-a analysis.
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 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/cesi-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
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 %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 (Tweets, 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 doses, 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.

(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.
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 evalu-
ation, 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 \(\text{kg}^{-1}\text{-day}^{-1}\) were not consid-
ered 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 \(\text{kg}^{-1}\text{-day}^{-1}\) and, to a greater extent, 1,000 mg \(\text{kg}^{-1}\text{-day}^{-1}\) caused
histopathological findings in the centrilobular region of the liver charac-
terized 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
\(\geq 500\) mg \(\text{kg}^{-1}\text{-day}^{-1}\) for \(\geq 3\) 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 \(\geq 500\) mg \(\text{kg}^{-1}\text{-day}^{-1}\) and statistically significant findings at 1,000 mg \(\text{kg}^{-1}\text{-day}^{-1}\) with dosing
\(\geq 14\) days. These histopathology and clinical chemistry biomarkers sup-
port 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 \(\text{kg}^{-1}\) 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-\((\leq 1,000\) mg \(\text{kg}^{-1}\text{-day}^{-1}\)) treated rats treated up to
29 days.

5  |  CONCLUSION

Acetaminophen when administered at dose levels of 250, 500, 1,000,
or 2,000 mg \(\text{kg}^{-1}\text{-day}^{-1}\) 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 assess-
ments nor in the percentage of micronucleated RETs were observed
except in the high dose group of 1,000 mg \(\text{kg}^{-1}\text{-day}^{-1}\) at hematotoxic
exposures. At 1,000 mg \(\text{kg}^{-1}\text{-day}^{-1}\), max 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 therapeu-
tic 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 \(\text{kg}^{-1}\text{-day}^{-1}\) 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
erthropoiesis. The small increase in the %MN-RETs should be carefully
interpreted given that it is not uncommon to see micronucleus induc-
tion at treatment levels which impact erythropoiesis. Changes in eryth-
ropoiesis 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 parame-
ters, 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.

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CONFLICT OF INTEREST

All authors were employees of The Janssen Pharmaceutical Compa-
nies 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.

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SUPPORTING INFORMATION

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

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