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Comparison of integrated genotoxicity endpoints in rats after acute and subchronic oral doses of 4-nitroquinoline-1-oxide

Daniel J. Roberts^{1,2}, Marie McKeon^{1,3}, Yong Xu^{1,3}, Leon F. Stankowski Jr.^{1,3}

¹Covance Laboratories, Inc., Vienna, VA, USA

²Bristol-Myers Squibb, New Brunswick, NJ, USA (current address)

³BioReliance Corporation, Rockville, MD, USA (current address)

Abstract

During interlaboratory validation trials for the *Pig-a* gene mutation assay we assessed the genotoxicity of 4-nitroquinoline-1-oxide across endpoints in multiple tissues: induction of *Pig-a* mutant red blood cells (RBCs) and reticulocytes (RETs); micronucleated RETs (MN RETs); and DNA damage in blood and liver via the alkaline Comet assay (%tail intensity [TI]). In a previous subchronic toxicity study with 28 daily doses, biologically meaningful increases were observed only for *Pig-a* mutant RBCs/RETs while marginal increases in the frequency of MN RET were observed, and other clastogenic endpoints were negative. Follow up acute studies were performed using the same cumulative doses (0, 35, 70, 105, and 140 mg/kg) administered in a bolus, or split over 3 equal daily doses, with samples collected up to 1 month after the last dose. Both of the acute dosing regimens produced similar results, in that endpoints were either positive or negative, regardless of 1 or 3 daily doses, but the 3 consecutive daily dose regimen yielded more potent responses in TI (in liver and blood) and *Pig-a* mutant frequencies. In these acute studies the same cumulative doses of 4NQO induced positive responses in clastogenic endpoints that were negative or inconclusive using a subchronic study design. Additionally, a positive control group using combination doses of cyclophosphamide and ethyl methanesulfonate was employed to assess assay validity and potentially identify a future positive control treatment for integrated genetic toxicity studies.

Keywords

Pig-a; micronucleus; comet assay; 4-nitroquinoline-1-oxide (4NQO)

CORRESPONDING AUTHOR: LFSJr, BioReliance Corporation, 14920 Broschart Road, Rockville, MD 20850, Tel: 240-447-2674, FAX: 301-738-2362, leon.stankowski@bioreliance.com.

STATEMENT OF AUTHOR CONTRIBUTIONS

LFS designed the experiments and was the principal investigator for the *Pig-a* gene mutation assay. YX was the principle investigator for the Comet and MN RET assays. MM directed the integrated rat genetic toxicity studies. DJR assisted with study design, was the flow cytometrist, and primarily wrote the manuscript with feedback from co-authors. Covance and BioReliance are contract research organizations that may offer the services described within.

INTRODUCTION

The field of genetic toxicology has historically served to assess the potential hazard(s) that xenobiotics may have on the integrity, fidelity, or stability of the genome by conducting appropriate *in vitro* and *in vivo* assays [Cimino, 2006]. As the field continues to evolve, weight-of-evidence strategies are employed to interpret data obtained from the standard battery of tests and *in vivo* data are generally used to understand safety margins based on point of departure analyses [Gollapudi, et al. 2013; Johnson, et al. 2014]. This creates a need for assays that reduce animal use and generate data that are acquired rapidly and objectively. Chromosomal damage (*i.e.*, clastogenicity and aneugenicity), can be detected by measuring induction of micronuclei in bone marrow or peripheral blood reticulocytes (RETs) with automated data collection platforms such as flow cytometry or image analysis. This assessment can be added to existing toxicology studies, which reduces overall animal use and has the benefit of shipping samples to test sites for analysis. The *Pig-a* gene mutation assay has similar benefits in that it also is a blood-based assay allowing integration into ongoing toxicology studies, samples can be analyzed across test sites, and mutant cells can be rapidly enumerated via flow cytometry [Dertinger, et al. 2014; Dobrovolsky, et al. 2010; MacGregor, et al. 2015]. While the Comet assay requires dedicated animals at necropsy due to tissue/organ requirements, sample fidelity has been maintained after freezing and shipping across test sites [Recio, et al. 2012]. Hence, all endpoints used herein can be easily integrated into routine toxicity testing if scientifically warranted.

The *Pig-a* gene mutation assay has been shown to predict chemicals that are known rodent carcinogens, and despite measuring micronuclei and *Pig-a* mutant frequencies (MFs) from the same hematopoietic tissue, they provide different information reflective of the different modes of action (MOAs) responsible for initiating the carcinogenic process [Dertinger, et al. 2012; MacGregor, et al. 2015]. The unique advantage of the Comet assay is being able to measure DNA damage in any tissue or organ, where generally site of first contact and liver (for metabolite assessment) are assessed [Uno, et al. 2015]. When considering integrating genetic toxicity evaluations into ongoing *in vivo* assays, it is important to understand the factors that influence the ability to accurately and precisely measure each response. To date a number of publications have focused on this, with many addressing the comparability of genetic toxicity endpoints using integrated study designs [Bhalli, et al. 2011b; Cammerer, et al. 2011; Cao, et al. 2014; Dobrovolsky, et al. 2013; Lemieux, et al. 2011; Lynch, et al. 2011; McDaniel, et al. 2012; Shi, et al. 2011; Stankowski, et al. 2011]. The current format of the *Pig-a* gene mutation assay is capable of detecting mutagens using acute or subchronic dosing, and the induction of the mutant phenotype has been shown to be additive for multiple doses of model mutagens, as well as to persist for some agents such as N-ethyl-N-nitrosourea (ENU [Miura, et al. 2009]). The work presented here was conducted to better characterize the relatively weak positive response obtained for 4-nitroquinoline-N-oxide (4NQO) using a 1 month dose regimen [Stankowski, et al. 2011], and also to evaluate the persistence and magnitude of responses in various genetic toxicity endpoints using different dosing and sampling strategies. These results highlight technical and biological considerations for designing dedicated genetic toxicity studies, or integrating genetox

endpoints into longer term toxicity studies, which generally use lower (exposure-relevant) daily doses.

MATERIALS AND METHODS

Reagents

Prototype MutaFlow[®] Rat *Pig-a* Mutation Assay Kits and MicroFlow[®] Rat Blood Micronucleus Kits were obtained from Litron Laboratories (Rochester, NY). 4NQO, cyclophosphamide (CP), dimethyl sulfoxide (DMSO), ethidium bromide, ethylenediaminetetraacetic acid (EDTA), ethyl methylsulfonate (EMS), methylcellulose, sodium hydroxide (NaOH), and Tris-HCL were supplied by Sigma-Aldrich (St. Louis, MO). Lympholyte[®] Mammal Solution was obtained from Cedarlane (Burlington, NC). Phosphate-buffered saline (PBS) and Hank's balanced salt solution (HBSS) were obtained from Media-Tech (Manassas, VA). Fetal bovine serum (FBS) was acquired from Quality Biological, Inc. (Gaithersburg, MD) and Comet assay supplies (low-melting agarose, lysis solution and 2-well slides) were acquired from Trevigen (Gaithersburg, MD).

Test Facility and Animal Husbandry

Sprague Dawley[®] male rats (6–8 weeks old, Harlan; Frederick, MD) were housed singly in wire bottom cages and acclimated for five days prior to the start of the study. Certified Rodent Diet (#2016C; Harlan Laboratories, Inc.; Indianapolis, IN) and water were provided *ad libitum*, except when fasted overnight prior to sacrifice. These studies were conducted at Covance Laboratories, Inc. in 2010, and a 12-hour light/dark cycle was used while maintaining room temperature between 18 and 26 °C. General daily clinical observations as well as weekly detailed observations were performed for each animal. All practices followed approved standard operating procedures and were in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

4NQO and CP/EMS Treatment

The daily doses administered in the 1 month study (0, 1.25, 2.5, 3.75, and 5 mg/kg daily x 28) have been previously reported [Stankowski, et al. 2011]. In two new studies reported herein, rats were given the same cumulative dose (28 times previous daily doses) as either a single bolus (0, 35, 70, 105, and 140 mg/kg) or in equal fractions for 3 consecutive days (0, 11.7, 23.3, 35, and 46.7 mg/kg daily x 3). Since the maximum dose initially evaluated in the 1 month study was not well tolerated (7.5 mg/kg/day [mkd]), an additional lower cumulative dose of 17.5 mg/kg was included in these acute studies (17.5 mg/kg in a single bolus dose or 3 doses of 5.8 mkd). Dose groups were increased to 10 rats (for the high dose 20, due to expected toxicity from the 1 month study) and half of each dose group was sacrificed 3 hours after the last dose for Comet liver evaluations, while recovery animals were used to collect longitudinal blood based data. Dose formulations were prepared daily in 0.5% (w/v) methylcellulose (Sigma-Aldrich; St. Louis, MO) in reverse osmosis deionized water and administered by oral gavage within 3 hours of preparation. All dose formulations were corrected for potency, and the first day of dosing was designated as day 1. To qualify the 1

and 3 day assays, additional groups of animals (N=3) were administered positive control articles CP and EMS in the same fashion; single combined bolus dose of 30 mg/kg CP plus 200 mg/kg EMS, or 10 mg/kg CP plus 66.7 mg/kg EMS daily, for 3 consecutive days. In addition to exploring additivity of the CP/EMS combination doses, the goal was to identify potential doses of routinely used positive control articles that could be utilized in an integrated genetic toxicity study that would be positive across all endpoints evaluated.

Blood and Tissue Collection

Minimal volumes of blood were collected by jugular venipuncture at the specified timepoints (Table I). Generally, 100 μ L was collected at each timepoint into spray coated heparin microtainers (BD; Franklin Lakes, NJ). Samples were kept at ambient temperature for *Pig-a* gene mutation and MN RET evaluations, while the Comet samples were collected on ice and immediately processed. Tissue and blood samples for the Comet assay were collected 3 hours after the last dose, which was staggered to ensure accurate necropsy timing. Collection and processing of the samples was highly choreographed and rapid, due to the labile nature of the Comet samples (described below in the Comet methods section).

Pig-a Gene Mutation Assay

Blood was processed, labeled, and analyzed according to the instructions received with the Prototype MutaFlow[®] Rat *Pig-a* Mutation Assay Kit. Approximately 350,000 RETs and 1 million red blood cells (RBCs) were analyzed to quantify the induced CD59-negative mutant phenotype per animal and timepoint. A FACSCanto II flow cytometer with FACSDiva v6.1.1 software (BD Biosciences; San Jose, CA) was used for data acquisition and analysis.

MN RET Assay

Blood was processed, labeled, and analyzed according to the instructions received with the Microflow[®] Rat Blood Micronucleus Kit. Approximately 20,000 RETs were evaluated for micronuclei per animal and timepoint, where available. A FACSCanto II flow cytometer with FACSDiva v6.1.1 software was used for data acquisition and analysis.

Alkaline Comet Assay

The alkaline Comet assay was used to detect DNA damage in rat peripheral blood and liver as previously described [Stankowski, et al. 2011]. The dose administration was staggered to facilitate precise timing of tissue collection, which reduced the time it took to immerse cells in agar, thereby minimizing potential sample degradation. Briefly, liver was rinsed in ice-cold Merchant's solution, minced, and 15 μ L of single cell preparation was added to 300 μ L 0.7% (w/v) low melting point agarose prior to slide preparation. Three 2-well slides were prepared and after applying coverslips, gels were solidified in a refrigerator. After hardening, the coverslips were removed and slides were placed in lysis buffer overnight, refrigerated and protected from light. All slides were unwound in refrigerated alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 30 minutes, and 2 of the 3 slides were electrophoresed at 0.7 V/cm for 30 minutes, under refrigerated conditions. Neutralized slides were dried and stored at room temperature prior to staining DNA with 20 μ g/mL ethidium bromide and acquiring data using fluorescence microscopy and an image analysis system.

(Comet Assay IV; Perceptive Instruments; Haverhill, UK). At this point in the development of the Comet assay, one hundred cells per tissue were evaluated for each animal, which was in accordance with accepted criteria at the time of study conduct [Tice, et al. 2000]. Median %tail intensity (TI) was calculated from the 100 cells and group averages were reported.

Statistics and Data Analysis

For the *Pig-a* gene mutation assay, up to 1 million cells were scored to enumerate total RBC MFs, but only 350,000 RETs were scored to measure MFs in the younger erythrocyte population. At the time of assay validation this was practical and considered valid [Dertinger, et al. 2011b]. However, the ability to precisely measure basal or weakly induced MFs, especially for RETs, was impacted by frequent zeros in the *Pig-a* MF data set. To address this, data analysis began with assessment of variance homogeneity across groups using Levene's test. Generally, variance was homogeneous after rank transformation, so transformed data were analyzed across all endpoints to maintain consistency of the statistical evaluation. If analysis of variance (ANOVA) was significant ($p < 0.05$, SAS v9.1), *post hoc* Dunnett's t-tests were conducted to determine pairwise significance (only compared to the corresponding vehicle control group [two-tailed, $p < 0.05$]). All data points represent the average result (for 5 animals, except for the highest dose level ($n=10$, in case of mortality) and positive control groups ($n=3$, to reduce overall animal use). Standard error of the mean (SEM) was used to evaluate how well the sample mean estimated the population mean. Upper 95% control limits (95% UCL) were defined using untreated and/or vehicle-treated historical control means plus 2 standard deviations, which were used in combination with the results from Dunnett's t-test to independently assess the biological significance of response for each endpoint.

RESULTS

RBC *Pig-a* Gene Mutation Assay (1 million cells scored)

Statistically significant, dose-dependent increases in RBC MFs were observed at 35 mg/kg both 2 and 4 weeks after a single oral dose of 4NQO (Figure 1A). The magnitude of response was similar between 1 and 3 daily treatments (Figure 1B). After 4 weeks, the high dose MF's induced after acute treatment were approximately 2-fold higher than the MF observed after fractionating the dose over a 1 month period (Figure 1C). Induced RBC MFs persisted over the course of the recovery periods, with the 3 daily dose regimen inducing slightly higher MFs at 70 mg/kg 4 weeks after treatment (Figure 1B). Statistically significant increases in MFs also were observed 2 and 4 weeks after single combination doses of 30 mg/kg CP and 200 mg/kg EMS (Figure 1D). However, the increases at 2 weeks post dose were not above the 95% UCL for the RBC assay ($MF=5.99 \times 10^{-6}$), which did not meet our positive response criteria. Splitting these treatments into 3 equal daily doses induced comparable MFs, indicating that mutagenicity of CP and EMS, when combined, may be additive, as the SEM's overlap suggesting no significant differences between the treatment regimens [Cumming, et al. 2007].

RET Pig-a Gene Mutation Assay (350,000 cells scored)

Dose-dependent increases in MFs were observed at 35 mg/kg following a single dose of 4NQO, and responses observed at 70 mg/kg were statistically significant (Figure 2A). The magnitude of the response and the shape of the dose-response curves were similar between 1 and 3 daily treatments, though the latter dosing regimen yielded statistically significant results by week 4 at 35 mg/kg (Figure 2B). Similar to observations in the RBC pool, administering 140 mg/kg of 4NQO cumulatively over 3 days induced higher mean MFs than a single bolus dose administration (42% and 59% higher at 2 and 4 weeks post dose, respectively). By week 4, both of the acute treatment protocols induced higher MFs than the 1 month study design (Figure 2C). In contrast to the statistically significant increases in RBC MFs 2 weeks after acute doses of CP/EMS, no statistically significant increases in RET MFs were observed after a single dose (2 and 4 weeks post dose) or 2 weeks after 3 daily doses. For the 3 daily dose regimen (week 2), this is likely due to scoring only 350,000 RET, which was practical at the time, and consequently impacted the precision of measuring MFs under 10×10^{-6} . While the group mean (14.2×10^{-6}) was above the 95% UCL (8.7×10^{-6}), an animal in the vehicle control group had a RET MF of 8.6×10^{-6} , and after rank transformation, the ANOVA indicated no significant variation between vehicle and positive control treatments: $F(1,6)=2.9$, $p=0.14$. Hence, a *post hoc* pairwise comparison was not conducted and since both positive response criteria were not met, the elevated group mean MF was considered only marginally increased. However, by week 4 the RET MF observed after 3 equal daily doses of CP/EMS was statistically significant and was higher than the 95% UCL, which was considered biologically meaningful and supports use of the CP/EMS combination as potential positive controls for this integrated study design. The induced MFs in RETs after CP/EMS dose administration did not appear to be additive (Figure 2D), which differs from observations in the total erythrocyte pool (Figure 1D).

MN RET Assay

Statistically significant, dose-dependent increases in the frequency of MN RETs were observed on day 3 at doses 35 mg/kg when administered as a single dose, and 70 mg/kg when split into 3 days or 1 month of dosing (Figure 3A). When using the acute dosing regimens, MN RET frequencies for 4NQO were orders of magnitude higher than the MN RET frequencies observed after administering equivalent cumulative doses of 4NQO fractionated over 1 month. The latter reached only a 2-fold increase at 140 mg/kg, which is this assay's threshold for detecting a positive response with adequate power [Kissling, et al. 2007]. In contrast, at 140 mg/kg the 1 and 3 daily dose regimens induced 17- and 9-fold increases in MN RET, suggesting the acute treatment regimens revealed the clastogenic potential of 4NQO. Statistically significant increases in the frequency of MN RETs also were observed after single or 3 consecutive daily CP/EMS treatments, with similar magnitudes of response (Figure 3B).

Alkaline Comet Assay (Blood)

Statistically significant increases in group mean TIs were observed 3 hr after single doses of 70 and 140 mg/kg, but not for the 105 mg/kg dose group ($p=0.16$ [Figure 4A]). There was evidence for a dose-response (linear regression; $p < 0.05$) and the maximum group response

(mean 2.91%) was observed at the high dose, which was above the 95% UCL for this tissue (1.58%). Hence, considering all these factors, the response appears positive but is barely above the 95% UCL. Two weeks later, statistically significant increases in TI were observed at 105 mg/kg (1.99%) and 140 mg/kg (1.07% [Figure 4B]). Again, there was evidence for dose-response (linear regression; $p = 0.05$) but the maximum response was not observed at the high dose, the high dose group mean was below the 95% UCL, and the lower estimate of the 105 mg/kg group mean (TI=1.6%), which drove the apparent dose-response, nearly overlaps with the 95% UCL. Considering the transient nature of TI induction, and that by 4 weeks there were no statistically significant increases (Figure 4C), the elevated response observed 2 weeks after treatment were likely a consequence of low and precise TI in the vehicle control group and were not indicative of persisting damage [Lorenzo, et al. 2013]. In contrast to the single dose administration, when splitting the dose over 3 daily administrations, clear dose-dependent increases in TI were observed 3 hours post dose, and they reached statistical significance at 105 mg/kg (Figure 4A). Two and 4 weeks after treatment, group mean TIs were below 3% for all doses and were not statistically significant (Figure 4B–C). Statistically significant increases in TI were observed 3 hours after single or 3 daily CP/EMS treatments, and they returned to basal frequencies by week 4, which is similar to the kinetics observed after single doses of 4NQO (Figure 4D).

Alkaline Comet Assay (Liver)

A statistically significant 2-fold increase in group mean TI was observed 3 hours after a single dose of 140 mg/kg 4NQO (Figure 5A). When splitting the doses over 3 days of treatment, statistically significant increases in TI were observed at doses ≥ 35 mg/kg. However, only the maximum dose (140 mg/kg) was clearly elevated, suggesting a potential threshold effect (Figure 5A). As expected, after 1 month recovery post single or 3-daily doses, no increases in liver TI were observed (data not shown). When fractionating the cumulative dose over the course of 1 month, no increase in TI was observed, which indicates any damage that occurred at these lower daily doses was not detectable 24 hours after the last dose administration. There were statistically significant increases in TI 3 hours after single or 3 day CP/EMS treatments (Figure 5B). All statistically significant findings for liver tissue were considered biologically meaningful as the group mean responses exceed the 95%UCL for this tissue (2.42%).

DISCUSSION

Pig-a Gene Mutation

Fractionating the 140 mg/kg dose over 1 month (5 mkd x 28) induced MFs that were approximately 2-fold lower than the acute treatments (Figures 1 and 2). This is likely attributed to the lack of a full expression period as newly established non-lethal mutations in bone marrow erythroid cells would not be expressed in the periphery until after the mutants matured, differentiated, and entered systemic circulation (Dzierzak and Philipsen 2013). In this study, it appears that a potential steady state for mutant phenotype cells was observed in as little as 2-weeks after the final dose. This is demonstrated by the general overlap in the dose response curves 2 and 4 weeks after acute treatments (Figures 1 and 2) and supports that the mutant induction observed after 1 month of daily dosing was not maximal, as the

last few weeks of induced mutants were not observable at the final time point. A similar 2-fold difference in response between acute and fractionated treatments at similar timepoints using identical analytical methods has been described for EMS, another alkylating agent [Dobo, et al. 2011].

The *Pig-a* MFs observed after administering 35 mg/kg 4NQO in a single or 3 day split dose were comparable to previous findings in Wistar rats [Phonethepswath, et al. 2010]. By week 4 of the present study, group mean RBC MFs from each acute dosing regimen ranged from 4.8 to 10.4×10^{-6} after 35 mg/kg 4NQO exposure (Figure 1A–B). At the same timepoint, Phonethepswath and colleagues observed a mean RBC MF of approximately 8×10^{-6} after 3 daily 4NQO administrations of 12.5 mg/kg (cumulatively 37.5 mg/kg). The similarity of response suggests that induction of *Pig-a* mutant erythrocytes is additive for 4NQO when using an acute dosing regimen, especially considering measurements at both laboratories were made prior to the introduction of an enrichment strategy that increases the precision of MF measurements in this range [Dertinger, et al. 2011a]. Hence, the magnitude of response was near the lower limits of assay sensitivity at the time. When evaluating data collected during week 4 of the 1 month study, doses 105 mg/kg failed to produce a positive response. Considering that the acute dosing regimens were positive at 35 mg/kg, and 2 weeks into the 1 month study the 105 mg/kg dose group had a total exposure of 52.5 mg/kg, we would expect mutant induction from the first 2 weeks to be detected by week 4. However, additivity of response may be dependent upon overcoming daily metabolic and excretory capacity, as 3.75 mkd is a much lower daily burden, and with additional dosing during the 2 weeks following this exposure, additional toxicity may have impacted our ability to detect increases in mutant cells. This is in agreement with a report in rats using the higher powered version of the *Pig-a* gene mutation assay, where 3 mkd was negative after 1 month of daily dose administrations [Zhou, et al. 2014]. In an equally powered assay to the format used herein, where 1 million RBC from 5 animals are evaluated for mutant phenotype, higher single doses of 50 and 100 mg/kg 4NQO induced near linear MFs that were approximately 2-fold apart in magnitude [Kimoto, et al. 2012]. A similar response was observed here after single doses of 70 and 140 mg/kg, even when using different antibodies and gating logic to identify and enumerate mutant phenotype cells (Figure 1a; group mean RBC MFs were 15.5 and 34.2×10^{-6} , respectively). Hence, these data support additivity of mutant induction with 4NQO, provided there is adequate expression time for MFs to reach steady state, and that the daily dose exceeds the threshold for mutant cell induction and detection using this assay.

Recently, a lack of response was observed when measuring *Pig-a* mutant erythrocytes in *gpt* transgenic mice after a single oral administration of 50 mg/kg 4NQO [Horibata, et al. 2013]. This exposure would approximate the single dose administration of 35 mg/kg used herein after allometric scaling [Klaassen, et al. 2007]. Surprisingly, using a similarly powered assay in wild type rats, we were able to detect an increase in RBC MF when evaluating the same number of cells (~1 million) at similar timepoints after exposure. Generally induction of this mutant phenotype has been observed to be more rapid and less persistent in mice [Bhalli, et al. 2011a]. However, samples were collected at identical post-dose intervals, which actually favored the kinetics of response manifestation in mouse based on the more rapid turn-over rate of RBCs in this species. The difference in response may be attributed to saturating DNA repair capacity but likely arises from species specific

pharmacokinetic differences. For example, 30 mg/kg 4NQO has the ability to induce structural chromosome aberrations in mouse bone marrow cells after a single intraperitoneal (IP) injection, indicating that the target tissue is susceptible to genotoxicity [McFee, 1989]. Since NQO1 is expressed in multiple tissues, IP injection circumvents first-pass metabolism and, as a consequence, parent 4NQO is reduced and metabolized to proximate genotoxins within the target tissue [Casado, et al. 2011; Iskander, et al. 2006; Tada, 1976]. Hence, a predominant fraction of 4NQO is hepatically metabolized after oral exposure to genotoxic intermediates that react at site of formation. This is supported by the observation that *gpt* MFs measured in the *gpt* mice were elevated and statistically significant in liver tissue but were similar to baseline frequencies in the bone marrow [Horibata, et al. 2013]. Reportedly, there is an approximate 10-fold difference in hepatic NQO1 baseline activity between mouse and rat, which may also contribute to the different *Pig-a* MFs observed in these rodent species [Stiborova, et al. 2014; Yates, et al. 2007]. Hence, the pharmacokinetic fate of the chemical under investigation, and metabolic differences in test systems, should be considered to ensure target tissue exposure.

Micronucleus Induction

Acute 4NQO treatments induced dose-related increases in MN RETs that reached statistical significance at 35 and 70 mg/kg (cumulative dose) with 1 or 3 daily treatments, respectively (Figure 3A). The 2-fold disparity in dose-effect could be attributed to collecting blood on day 3 for both dosing regimens. Kinetically, this was optimal for the single dose administration, as highest magnitude of response has been shown with other genotoxins 48 hours after a single acute treatment [MacGregor, et al. 2006]. However, the MN RET frequencies measured in peripheral blood on the third day after 3 daily doses may not be maximal as the response is likely approaching steady state [Hayashi, et al. 2000]. These results were similar to a report using Wistar rats where 3 daily administrations of 4NQO totaling 75 mg/kg induced statistically significant increases in MN RETs but a cumulative dose of 36.5 mg/kg was only elevated and considered negative [Phonethepswath, et al. 2010]. It appears that the induction of MN RETs was clearly detected only when utilizing acute treatment protocols, favoring a single bolus dose. When fractionating 140 mg of 4NQO over 1 month in equal daily doses, a small increase in MN RETs has been reported as negative [Dertinger, et al. 2010] and was deemed questionable in our hands [Stankowski, et al. 2011], as the minimum 2-fold increase required for a *bona fide* positive response was only met and not exceeded [Kissling, et al. 2007]. While there was no clear MN RET induction in blood during the 1 month studies, mutant cell induction in the same tissue was identifiable (Figures 1C and 2C). Hence, when integrating genetic toxicity endpoints into a subchronic study design that employs lower doses, the predominant genotoxic MOA should dictate which endpoint(s) are appropriate, and should be known from previous *in vitro* studies.

Comet Evaluation

The most contrasting acute responses were observed in blood and liver when evaluating DNA damage using the alkaline Comet assay (Figures 4 and 5). The response measured after a single dose of 140 mg/kg 4NQO was considered positive in both blood and liver but was approximately 50% lower in magnitude than the response observed after fractionating

the same dose over 3 days. For liver, each acute treatment regimen produced clearly elevated results only at the high dose (Figure 5A). Also, after fractionating the 140 mg/kg dose over a month of oral administrations, there were no statistical or biologically meaningful increases in TI. However, the sampling interval for the 1 month study was 24 hours after the last dose and any transient damage may have been repaired by the sampling timepoint. 4NQO has induced TI in multiple organs of the mouse 3 and 8 hours after a single oral dose of 200 mg/kg, suggesting some persistence of damage after high acute treatments [Sasaki, et al. 2000]. Overall for this predominantly mutagenic compound, the 3 daily dose study design seems to be the best at demonstrating 4NQOs clastogenic potential. It is likely that the rate of DNA repair, metabolism, and excretion were adequate to ameliorate structural DNA damage when using lower doses in a protracted study design. Unlike induction of MN RETs, which was highest 2-days after a single dose, the Comet response in blood and liver was highest 3 hours after 3 daily administrations. Since formation of micronuclei is reliant upon cellular division this observation may be attributed to bone marrow being more labile while the cells scored in the comet assays (hepatocytes and circulating leukocytes) are more stable. Regardless, TI induction in blood was also more easily detected after 3 daily doses, which may be due to more parent 4NQO reaching the bone marrow when using multiple exposures in a short period of time. Alternately, the magnitude of TI induction after 3 days of dosing could have been increased by the amount of oxidative stress caused by redox cycling, which would increase TI due to oxidation of bases that effectively saturated real time DNA repair capacity [Nunoshiba, et al. 1993]. Kinetically, the DNA damage measured *via* the alkaline Comet assay returned to basal levels during the recovery period, which is expected as the lesions are repaired [Lorenzo, et al. 2013]. Overall, the Comet endpoint appeared to be the least sensitive in terms of magnitude of response, with liver being more sensitive than blood. This is not alarming as 4NQO predominantly elicits genotoxicity (and subsequent carcinogenicity) after biotransformation (NQO1 reduction) via a mutagenic, not clastogenic, MOA. While there is an expectation to conduct appropriate batteries of *in vivo* genotoxicity tests, with the current availability of *in vitro* and *in silico* techniques that can elucidate predominant MOA, information on the mechanism of potential genotoxicity should guide selection of *in vivo* genotoxicity endpoints, regardless of the intended xenobiotic use (pharmaceutical, herbicidal, industrial chemical, etc.).

CP/EMS Treatment

A small positive control treatment group (n=3) was included to ensure assay validity and assess the ability of typical positive control articles to elicit positive responses in all 3 endpoints, across 2 tissues, after acute treatment. Utility of these agents to induce positive responses in the MN RET and Comet assays was successful, as clear elevations of damage were detected (Figures 3B, 4D, and 5B). This was not unexpected as CP (30 mg/kg) and EMS (200 mg/kg) are typical positive control articles (and doses) routinely used for these endpoints. The weakest response was induction of RET and RBC MFs after 3 daily doses, which was only clearly positive 1 month after treatment (Figures 1D and 2D). In a similarly powered assay where 1 million cells were sampled, CP has been shown to be a weak inducer of RBC MFs for cumulative doses of 50 and 80 mg/kg [Bhalli, et al. 2013 and Kimoto, et al. 2012]. Hence, with only administering 30 mg/kg CP, EMS was considered responsible for inducing the majority of RBC and RET MFs. This is supported by work

using an enrichment strategy that greatly increases the numbers of cells scored [Dertinger, et al. 2012], which increases the precision of MFs in this range. In this report, clear increases in RET and RBC MFs were observed 2 weeks after treatment with a cumulative dose of 45 mg/kg CP but not 22.5 mg/kg (the latter was statistically significant in RETs, though marginally increased). Hence, in the studies presented herein, 30 mg/kg CP would not be expected to appreciably contribute to the RET and RBC MFs. However, EMS alone at 200 mg/kg (cumulatively) may also not be adequate in driving the induction of *Pig-a* MFs. For example, when using a 1 month study design higher EMS doses were needed to elicit meaningful MFs, which suggests that co-administration of CP and EMS at these doses potentiated the genotoxicity [Gunther, et al., 2014]. In this report, positive responses in RETs and RBCs were observed 1 month after dosing 12.5 mkd and 25 mkd, respectively (cumulatively, 350 and 700 mg/kg EMS). Similar responses were also observed using a different labeling strategy and an acute study design with 360 mg/kg EMS [Kimoto, et al. 2012]. Also, similar patterns of where acute treatment induced a higher magnitude of RET and RBC MFs were reported for CP and EMS (independently) when sampling 2 and 4 weeks after treatment, respectively [Dobo, et al. 2011 and Dertinger, et al. 2012]. In both these studies, there was no co-administration with CP or EMS, so instead of potentiating mutagenicity, the lower protracted daily dosing regimens may have decreased the magnitude of exposure to the target tissue, due to daily metabolism, excretion, and DNA repair capacity. Considering the other genetic toxicity endpoints were clearly elevated with the CP/EMS combination dose, increasing the dose to ensure induction of adequate RET and RBC MFs may not be desirable, as toxicity (both hematopoietic and liver) can confound data interpretation.

CONCLUSION

Utilizing an acute regimen for 4NQO dose administration increased the amount of detectable genetic damage for all 3 endpoints studied herein. Standard toxicity tests are typically conducted at lower, exposure-relevant dose levels, which may underestimate genotoxic risk if evaluating only one type of genetic damage. In a 1 month study with low doses of 4NQO we reported increases in *Pig-a* MF, while the MN RET, chromosomal aberration, and Comet endpoints were clearly negative or questionable at best [Stankowski, et al. 2011]. When administering the same cumulative dose of 4NQO over shorter dose durations, the clastogenic effects of 4NQO became apparent and there was a greater magnitude of *Pig-a* mutant cell induction. Overall, the data presented herein further demonstrate that all genetic toxicity endpoints are not equivalent in terms of accumulation, persistence, and magnitude of response. Considering that 3 days of 4NQO (and CP/EMS) treatment produced positive responses in all endpoints across 2 target tissues, this study design may be optimal to detect both major genotoxic MOAs. If addressing positive *in vitro* genotoxicity test results, adding genotox endpoints to protracted toxicity study designs is not appropriate as the rates of absorption, metabolism, and elimination can prevent detection of a response, as target tissues and/or organs may not be highly exposed at low doses. Additionally, timing of sample collection for each endpoint is drastically different, as maximum responses from the *Pig-a* gene mutation, MN RET, and Comet assays were obtained 2 weeks, 48 hours post dose, and 3 hours post dose, respectively. Overall, the 3 day study design with 2 weeks recovery

could prove useful for assessing mutagenic, clastogenic, and aneugenic risk, when hazards have been identified using *in vitro* test systems.

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LITERATURE CITED

- Bhalli JA, Pearce MG, Dobrovolsky VN, and Heflich RH. 2011a Manifestation and persistence of Pig-a mutant red blood cells in C57BL/6 mice following single and split doses of N-ethyl-N-nitrosourea. *Environ Mol Mutagen* 52:766–773. [PubMed: 22167887]
- Bhalli JA, Shaddock JG, Pearce MG, Dobrovolsky VN, Cao X, Heflich RH, and Vohr HW. 2011b Report on stage III Pig-a mutation assays using benzo[a]pyrene. *Environ Mol Mutagen* 52:731–7. [PubMed: 22052432]
- Bhalli JA, Shaddock JG, Pearce MG, and Dobrovolsky VN. 2013 Sensitivity of the Pig-a assay for detecting gene mutation in rats exposed acutely to strong clastogens. *Mutagenesis* 28:447–55. [PubMed: 23677247]
- Cammerer Z, Bhalli J A, Cao X, Coffing SL, Dickinson D, Dobo KL, Dobrovolsky VN, Engel M, Fiedler RD, Gunther WC, Heflich RH, Pearce MG, Shaddock JG, Shutsky T, Thiffeault CJ, and Schuler M. 2011 Report on stage III Pig-a mutation assays using N-ethyl-N-nitrosourea-comparison with other *in vivo* genotoxicity endpoints. *Environ Mol Mutagen* 52:721–30. [PubMed: 22167886]
- Cao X, Mittelstaedt RA, Pearce MG, Allen BC, Soeteman-Hernandez LG, Johnson GE, Bigger CA, and Heflich RH. 2014 Quantitative dose-response analysis of ethyl methanesulfonate genotoxicity in adult *gpt*-delta transgenic mice. *Environ Mol Mutagen* 55:385–99. [PubMed: 24535894]
- Casado FL, Singh KP, and Gasiewicz TA. 2011 Aryl hydrocarbon receptor activation in hematopoietic stem/progenitor cells alters cell function and pathway-specific gene modulation reflecting changes in cellular trafficking and migration. *Mol Pharmacol* 80:673–82. [PubMed: 21791576]
- Cimino MC. 2006 Comparative overview of current international strategies and guidelines for genetic toxicology testing for regulatory purposes. *Environ Mol Mutagen* 47:362–90. [PubMed: 16649190]
- Cumming G, Fidler F, and Vaux DL. 2007 Error bars in experimental biology. *J Cell Biol* 177:7–11. [PubMed: 17420288]
- Dertinger SD, Phonethepswath S, Franklin D, Weller P, Torous DK, Bryce SM, Avlasevich S, Bemis JC, Hyrien O, Palis J, and MacGregor JT. 2010 Integration of mutation and chromosomal damage endpoints into 28-day repeat dose toxicology studies. *Toxicol Sci* 115:401–11. [PubMed: 20202993]
- Dertinger SD, Bryce SM, Phonethepswath S, and Avlasevich SL. 2011a When pigs fly: immunomagnetic separation facilitates rapid determination of Pig-a mutant frequency by flow cytometric analysis. *Mutat Res* 721:163–70. [PubMed: 21277384]
- Dertinger SD, Phonethepswath S, Weller P, Nicolette J, Murray J, Sonders P, Vohr HW, Shi J, Krsmanovic L, Gleason C, Custer L, Henwood A, Sweder K, Stankowski LF Jr., Roberts DJ, Giddings A, Kenny J, Lynch AM, Defrain C, Nessler F, van der Leede BJ, Van Doninck T, Schuermans A, Tanaka K, Hiwata Y, Tajima O, Wilde E, Elhajouji A, Gunther WC, Thiffeault CJ, Shutsky TJ, Fiedler RD, Kimoto T, Bhalli JA, Heflich RH, and MacGregor JT. 2011b International Pig-a gene mutation assay trial: evaluation of transferability across 14 laboratories. *Environ Mol Mutagen* 52:690–8. [PubMed: 21910140]
- Dertinger SD, Phonethepswath S, Avlasevich SL, Torous DK, Mereness J, Bryce SM, Bemis JC, Bell S, Weller P, and Macgregor JT. 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. *Toxicol Sci* 130:328–48. [PubMed: 22923490]

- Dertinger SD, Avlasevich SL, Bemis JC, Chen Y, and MacGregor JT. 2014 Human erythrocyte PIG-A assay: An easily monitored index of gene mutation requiring low volume blood samples. *Environ Mol Mutagen* 56:366–77. [PubMed: 25412990]
- Dobo KL, Fiedler RD, Gunther WC, Thiffeault CJ, Cammerer Z, Coffing SL, Shutsky T, and Schuler M. 2011 Defining EMS and ENU dose-response relationships using the Pig-a mutation assay in rats. *Mutat Res* 725:13–21. [PubMed: 21729764]
- Dobrovolsky VN, Miura D, Heflich RH, and Dertinger SD. 2010 The in vivo Pig-a gene mutation assay, a potential tool for regulatory safety assessment. *Environ Mol Mutagen* 51:825–35. [PubMed: 20857433]
- Dobrovolsky VN, Shaddock JG, Mittelstaedt RA, Miura D, and Heflich RH. 2013 Detection of in vivo mutation in the Hprt and Pig-a genes of rat lymphocytes. *Methods Mol Biol* 1044:79–95. [PubMed: 23896872]
- Dzierzak E, and Philipsen S. 2013 Erythropoiesis: development and differentiation. *Cold Spring Harb Perspect Med* 3:a011601. [PubMed: 23545573]
- Gollapudi BB, Johnson GE, Hernandez LG, Pottenger LH, Dearfield KL, Jeffrey AM, Julien E, Kim JH, Lovell DP, Macgregor JT, Moore MM, van Benthem J, White PA, Zeiger E, and Thybaud V. 2013 Quantitative approaches for assessing dose-response relationships in genetic toxicology studies. *Environ Mol Mutagen* 54:8–18. [PubMed: 22987251]
- Gunther WC, Coffing SL, Dickinson DA, Engel ME, Fiedler RD, O'Lone SD, Sanok KE, Thiffeault CJ, Shutsky TJ, Schuler MJ, and Dobo KL. 2014 Evaluation of the Pig-a, micronucleus, and comet assay endpoints in a 28-day study with ethyl methanesulfonate. *Environ Mol Mutagen* 55:492–9. [PubMed: 24599777]
- Hayashi M, MacGregor JT, Gatehouse DG, Adler ID, Blakey DH, Dertinger SD, Krishna G, Morita T, Russo A, and Sutou S. 2000 In vivo rodent erythrocyte micronucleus assay. II. Some aspects of protocol design including repeated treatments, integration with toxicity testing, and automated scoring. *Environ Mol Mutagen* 35:234–52. [PubMed: 10737958]
- Horibata K, Ukai A, Kimoto T, Suzuki T, Kamoshita N, Masumura K, Nohmi T, and Honma M. 2013 Evaluation of in vivo genotoxicity induced by N-ethyl-N-nitrosourea, benzo[a]pyrene, and 4-nitroquinoline-1-oxide in the Pig-a and *gpt* assays. *Environ Mol Mutagen* 54:747–54. [PubMed: 24105957]
- Iskander K, Li J, Han S, Zheng B, and Jaiswal AK. 2006 NQO1 and NQO2 regulation of humoral immunity and autoimmunity. *J Biol Chem* 281:30917–24. [PubMed: 16905546]
- Johnson GE, Soeteman-Hernandez LG, Gollapudi BB, Bodger OG, Dearfield KL, Heflich RH, Hixon JG, Lovell DP, MacGregor JT, Pottenger LH, Thompson CM, Abraham L, Thybaud V, Tanir JY, Zeiger E, van Benthem J, and White PA. 2014 Derivation of point of departure (PoD) estimates in genetic toxicology studies and their potential applications in risk assessment. *Environ Mol Mutagen* 55:609–23. [PubMed: 24801602]
- Kimoto T, Chikura S, Suzuki-Okada K, Kobayashi XM, Itano Y, Miura D, and Kasahara Y. 2012 Effective use of the Pig-a gene mutation assay for mutagenicity screening: measuring CD59-deficient red blood cells in rats treated with genotoxic chemicals. *J Toxicol Sci* 37:943–55. [PubMed: 23038003]
- Kissling GE, Dertinger SD, Hayashi M, and MacGregor JT. 2007 Sensitivity of the erythrocyte micronucleus assay: dependence on number of cells scored and inter-animal variability. *Mutat Res* 634:235–40. [PubMed: 17851117]
- Klaassen CD, and Casarett LJ. 2007 Casarett and Doull's Toxicology: The Basic Science of Poisons 7th Edition. McGraw-Hill Professional 1280 p.
- Lemieux CL, Douglas GR, Gingerich J, Phonetepswath S, Torous DK, Dertinger SD, Phillips DH, Arlt VM, and White PA. 2011 Simultaneous measurement of benzo[a]pyrene-induced Pig-a and lacZ mutations, micronuclei and DNA adducts in Muta Mouse. *Environ Mol Mutagen* 52:756–65. [PubMed: 21976233]
- Lorenzo Y, Costa S, Collins AR, and Azqueta A. 2013 The Comet assay, DNA damage, DNA repair and cytotoxicity: hedgehogs are not always dead. *Mutagenesis* 28:427–32. [PubMed: 23630247]

- Lynch AM, Giddings A, Custer L, Gleason C, Henwood A, Aylott M, and Kenny J. 2011 International Pig-a gene mutation assay trial (stage III): results with N-methyl-N-nitrosourea. *Environ Mol Mutagen* 52:699–710. [PubMed: 22167885]
- MacGregor JT, Bishop ME, McNamee JP, Hayashi M, Asano N, Wakata A, Nakajima M, Saito J, Aidoo A, Moore MM, and Dertinger SD. 2006 Flow cytometric analysis of micronuclei in peripheral blood reticulocytes: II. An efficient method of monitoring chromosomal damage in the rat. *Toxicol Sci* 94:92–107. [PubMed: 16888079]
- MacGregor JT, Frötschl R, White PA, Crump KS, Eastmond DA, Fukushima S, Guérard M, Hayashi M, Soeteman-Hernández LG, Johnson GE, Kasamatsu T, Levy DD, Morita T, Müller L, Schoeny R, Schuler MJ, Thybaud V. 2015 IWGT report on quantitative approaches to genotoxicity risk assessment II. Use of point-of-departure (PoD) metrics in defining acceptable exposure limits and assessing human risk. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* 873:66–78.
- McDaniel LP, Ding W, Dobrovolsky VN, Shaddock JG Jr, Mittelstaedt RA, Doerge DR, and Heflich RH. 2012 Genotoxicity of furan in Big Blue rats. *Mutat Res* 742:72–8. [PubMed: 22200623]
- McFee AF. 1989 Genotoxic potency of three quinoline compounds evaluated in vivo in mouse marrow cells. *Environ Mol Mutagen* 13:325–31. [PubMed: 2500337]
- Miura D, Dobrovolsky VN, Kimoto T, Kasahara Y, and Heflich RH. 2009 Accumulation and persistence of Pig-A mutant peripheral red blood cells following treatment of rats with single and split doses of N-ethyl-N-nitrosourea. *Mutat Res* 677:86–92. [PubMed: 19501187]
- Nunoshiba T, and Demple B. 1993 Potent intracellular oxidative stress exerted by the carcinogen 4-nitroquinoline-N-oxide. *Cancer Res* 53:3250–2. [PubMed: 8391920]
- Phonetheswath S, Franklin D, Torous DK, Bryce SM, Bemis JC, Raja S, Avlasevich S, Weller P, Hyrien O, Palis J, Macgregor JT, and Dertinger SD. 2010 Pig-a mutation: kinetics in rat erythrocytes following exposure to five prototypical mutagens. *Toxicol Sci* 114:59–70. [PubMed: 19965957]
- Recio L, Kissling GE, Hobbs CA, Witt KL. 2012 Comparison of Comet assay dose-response for ethyl methanesulfonate using freshly prepared versus cryopreserved tissues. *Environ Mol Mutagen* 53:101–13. [PubMed: 22069077]
- Sasaki YF, Sekihashi K, Izumiyama F, Nishidate E, Saga A, Ishida K, and Tsuda S. 2000 The comet assay with multiple mouse organs: comparison of comet assay results and carcinogenicity with 208 chemicals selected from the IARC monographs and U.S. NTP Carcinogenicity Database. *Crit Rev Toxicol*, 30:629–799. [PubMed: 11145306]
- Shi J, Krsmanovic L, Bruce S, Kelly T, Paranjpe M, Szabo K, Arevalo M, Atta-Safah S, Debelie F, LaForce MK, Sly J and Springer S. 2011 Assessment of genotoxicity induced by 7,12-dimethylbenz(a)anthracene or diethylnitrosamine in the Pig-a, micronucleus and Comet assays integrated into 28-day repeat dose studies. *Environ Mol Mutagen* 52:711–20. [PubMed: 21976072]
- Stankowski LF Jr, Roberts DJ, Chen H, Lawlor T, McKeon M, Murli H, Thakur A, and Xu Y. 2011 Integration of Pig-a, micronucleus, chromosome aberration, and Comet assay endpoints in a 28-day rodent toxicity study with 4-nitroquinoline-1-oxide. *Environ Mol Mutagen* 52:738–47. [PubMed: 22020836]
- Stiborova M, Levova K, Barta FX, Sulc M, Frei E, Arlt VM, and Schmeiser HH. 2014 The influence of dicoumarol on the bioactivation of the carcinogen aristolochic acid I in rats. *Mutagenesis* 29:189–200. [PubMed: 24598128]
- Tada M. 1976 Main binding sites of the carcinogen, 4-nitroquinoline 1-oxide in nucleic acids. *Biochim Biophys Acta* 454:558–66. [PubMed: 826278]
- Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, and Sasaki YF. 2000 Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen* 35:206–21 [PubMed: 10737956]
- Uno Y, Kojima H, Omori T, Corvi R, Honma M, Schechtman LM, Tice RR, Beevers C, De Boeck M, Burlinson B, Hobbs CA, Kitamoto S, Kraynak AR, McNamee J, Nakagawa Y, Pant K, Plappert-Helbig U, Priestley C, Takasawa H, Wada K, Wirmitzer U, Asano N, Escobar PA, Lovell D, Morita T, Nakajima M, Ohno Y, and Hayashi M. 2015 JaCVAM-organized international validation study of the in vivo rodent alkaline comet assay for detection of genotoxic carcinogens: II. Summary of definitive validation study results. *Mutat Res* 786–788:45–76.

- Yates MS, Tauchi M, Katsuoka F, Flanders KC, Liby KT, Honda T, Gribble GW, Johnson DA, Johnson JA, Burton NC, Guilarte TR, Yamamoto M, Sporn MB, and Kensler TW. 2007 Pharmacodynamic characterization of chemopreventive triterpenoids as exceptionally potent inducers of Nrf2-regulated genes. *Mol Cancer Ther* 6:154–62. [PubMed: 17237276]
- Zhou C, Zhang M, Huang P, Tu H, Wang Z, Dertinger SD, Torous DK, and Chang Y. 2014 Assessment of 5-fluorouracil and 4-nitroquinoline-1-oxide in vivo genotoxicity with Pig-a mutation and micronucleus endpoints. *Environ Mol Mutagen* 55:735–40. [PubMed: 25124805]

Fig. 1: Mutant RBC induction in rats after oral 4NQO dose administration. Data are represented as group means $\pm$ standard error of the mean. Induced mutant frequencies (A) before treatment (●), and 2 (■) or 4 (▲) weeks after a single bolus dose administration. Open symbols or * indicate $P < 0.05$ when compared with the respective vehicle control group. B : Cumulative dose split into three equal daily doses. C : Cumulative dose fractionated over 1 month in equal daily doses [Stankowski et al., 2011]. D : CP/EMS mutant RBC induction after 1 (gray) or 3 (black) doses. The 95% upper control limit (mean + 2 standard deviations) of vehicle-treated and basal mutant RBC frequencies (combined) was 5.99×10^{-6} .

Fig. 2:

Mutant RET induction in rats after oral 4NQO dose administration. Data are represented as group means $\pm$ standard error of the mean and is formatted identically to Figure 1. “NS” indicates lack of statistical significance after rank transformation, as the corresponding vehicle control group had an animal with an elevated MF (8.6×10^{-6}). The 95% UCL of vehicle-treated and basal mutant RET frequencies (combined) was 8.70×10^{-6} .

Fig. 3:

MN RET induction in rats after oral 4NQO dose administration. Data are represented as group means + standard error of the mean. A : Comparison of dose fractionation after a single dose (▲), three daily doses (●), and 28 daily doses (■). Open symbols or * indicate P 0.05 when compared to the respective vehicle control group. Collection of blood was on Day 3 for both the 1 and 3 dose regimens, and 24 h after 1 month of dosing [Stankowski et al, 2011]. B : CP/EMS MN RET induction after 1 or 3 daily doses, both sampled 48 h after the initial treatment. The 95% UCL of vehicle-treated MN RET frequencies was 0.125%.

Fig. 4:

DNA damage induction in rat blood after oral 4NQO dose administration using the alkaline Comet assay. Data are represented as group %tail intensity (TI, group mean of individual median TI) $\pm$ standard error of the mean. A : TI 3-hrs after a single dose (▲), 3 h after three daily doses (●), and 3 h after 1 month of daily doses (■ [Stankowski et al, 2011]). Open symbols or * indicate $P < 0.05$ when compared with the respective vehicle control group. B : 2-weeks and C : 4-weeks after the single or three daily dosing regimens. D : TI after 1 single (gray) or 3 consecutive daily (black) doses of CP/EMS. The 95% UCL of vehicle-treated TI was 1.58%.

Fig. 5:

DNA damage induction in rat liver after oral 4NQO dose administration using the alkaline Comet assay. Data are represented as group %tail intensity (TI, group mean of individual median TI) $\pm$ standard error of the mean. A : Comparison of dose fractionation using a single dose ($\blacktriangle$), three consecutive daily doses ($\bullet$), or 1 month of daily doses ($\blacksquare$). Livers were collected 3 h after the last dose for the 1 and 3 dose regimens, and 24 h after 1 month of dosing [Stankowski et al, 2011]. Open symbols or * indicate $P \leq 0.05$ when compared to the respective vehicle control group. B : CP/EMS TI induction after 1 (gray) or 3 (black) doses. The 95% UCL of vehicle-treated TI was: 2.42%.

Table I:

Sampling intervals, in terms of study day (and hours, as needed), where the first administration of 4NQO was designated as day 1.

No. of Daily Doses ^a	Comet Assay		Micronucleus Assay	<i>Pig-a</i> Gene Mutation Assay
	Liver	Peripheral Blood (PB)	PB	PB
1	1 (3hr) and 29	1 (3hr), 15, 29	3	-4, 15, 29
3	3 (3hr) and 31	3 (3hr), 17, 31	3	-1, 17, 31
28 ^b	29	1 (3hr) and 29	29	-1, 15, 29

^a All studies contained cumulative doses of 35, 70, 105, and 140 mg/kg 4NQO. The 1 and 3 day dosing regimens each included a lower cumulative dose of 17.5 mg/kg while the 1 month dosing schedule included a higher cumulative dose of 210 mg/kg.

^b The 1 month study is previously reported, and contained other sampling intervals not discussed herein. [Stankowski et al., 2011]