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Integration of liver and blood micronucleus and *Pig-a* gene mutation endpoints into rat 28-day repeat-treatment studies: Proof-of-principle with diethylnitrosamine

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A B S T R A C T

Regulatory guidance documents stress the value of assessing multiple tissues and the most appropriate endpoints when evaluating chemicals for *in vivo* genotoxic potential. However, conducting several independent studies to consider multiple endpoints and/or tissue compartments is resource intensive. Furthermore, conventional approaches for scoring genotoxicity endpoints are slow, tedious, and less objective than what would be considered ideal. In an effort to address these issues with current practices, we attempted to i) employ flow cytometry-based methods to score liver micronuclei, blood micronuclei, and blood *Pig-a* gene mutation, and ii) integrate the endpoints into a common general toxicology study design—the rat 28-day repeat dose study. A proof-of-principle experiment was performed with 6-week old male Crl:CD(SD) rats exposed to diethylnitrosamine (DEN) for 28 consecutive days. One day later blood was collected for micronucleated reticulocyte (MN-RET) and *Pig-a* mutation assays, and liver tissue was obtained for micronucleated hepatocyte (MNHEP) scoring. MN-RET frequencies were not affected by DEN exposure, and mean *Pig-a* mutant cell frequencies were only slightly elevated. On the other hand, % MNHEP showed marked, dose-related increases (2.2, 7.2, and 9.1 mean fold-increase for 5, 10, 15 mg DEN/kg/day, respectively). Concurrent with MNHEP analyses, assessments of Ki-67-positive events and the proportion of 8n nuclei provided evidence for treatment-related changes to hepatocyte proliferation. Collectively, these results reinforce the importance of evaluating chemicals' genotoxic potential in liver in addition to hematopoietic cells, and suggest that several automated measurements can be successfully integrated into repeat-dose studies for higher efficiencies and better utilization of fewer animals.

1. Introduction

The *in vivo* micronucleus test is most often conducted with immature erythrocytes (i.e., reticulocytes, or RET) obtained from bone marrow or peripheral blood [1–3]. Indeed, for the last several decades, the micronucleated reticulocyte (MN-RET) based assay has been a key component of regulatory safety assessment packages [4,5]. Even so, recent guidance documents stress the importance of considering other tissues in addition to those of the hematopoietic compartment. For example, the International Conference on Harmonisation S2(R1) guidance describes the importance of evaluating a second tissue to guard against negative MN-RET results being due to lack of adequate bone marrow exposure to a drug and/or its metabolites [4].

To address exposure and metabolite concerns, contemporary *in vivo* genotoxicity studies often supplement MN-RET analyses with a liver-based assay [6–8]. The liver is the main site of drug metabolism, and the concentration of genotoxic intermediates can therefore be highest in this tissue. However, the most commonly utilized approach, a liver tissue comet assay, is not sensitive to aneugens, and owing to fast repair kinetics, it is not easily integrated into standard repeat-dose toxicology studies. Industrial genetic toxicologists, regulatory officials, and other stake-holders are therefore in need of more efficient, 3Rs friendly methods for measuring *in vivo* DNA damage in non-hematopoietic tissues, especially the liver.

One potential solution that has been described by the JEMS/MMS work group is to treat 6-week old rats for 28 consecutive days, and one day later harvest liver tissue for micronucleated hepatocyte (MNHEP) measurements. This approach takes advantage of the fact that at this age the rate of rat hepatocyte proliferation has slowed but not ceased, and genotoxicant-induced MNHEP accumulate with repeat treatments

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[9,10]. Data collected to date are encouraging, as they suggest that as with MN-RET and *Pig-a* gene mutation assays [11–13], MNHEP analyses can be integrated into ongoing 28-day general toxicology studies [10,14–16].

The present report describes a proof-of-principle experiment aimed at enhancing the throughput and objectivity by which several genetic toxicology endpoints are made through the use of flow cytometry, while simultaneously addressing animal and other resource requirements by utilizing a 28-day integrated study design. The experiment was conducted with diethylnitrosamine (DEN), a potent hepatocarcinogen that has generated mixed results in the blood-based *Pig-a* mutation assay [17–19], and consistently negative results in MN-RET assays [17,18,20]. Thus, while many hepatocarcinogens that require enzymatic bioactivation in order to form proximate genotoxic and tumorigenic metabolites are positive in *Pig-a* and MN-RET assays [13,21], we anticipated DEN would highlight the desirability of investigating hepatocytes in addition to hematopoietic cells whenever adequate systemic exposure is not anticipated. The genotoxicity results, all generated *via* flow cytometric analyses, are discussed in terms of the desirability of higher data acquisition efficiencies, as well as the animal reduction opportunities afforded by commonly employed repeat-treatment study designs.

2. Materials and methods

2.1. Reagents, miscellaneous supplies

DEN (CAS No. 55-18-5) and dimethyl sulfoxide (CAS No. 67-68-5) were purchased from Sigma-Aldrich, St. Louis, MO. Heat-inactivated fetal bovine serum (FBS; cat. no. 89510-186) was from VWR, Radnor, PA. Reagents used for flow cytometric MN-RET scoring (Anticoagulant Solution, Buffer Solution, DNA Stain, Anti-CD71-FITC and Anti-CD61-PE Antibodies, RNase Solution, and Malaria Biostandards) were from *In Vivo* MicroFlow® PLUS R Kits, Litron Laboratories, Rochester, NY. Reagents used for flow cytometric enumeration of mutant erythrocytes (RBC^{CD59-}) and mutant reticulocytes (RET^{CD59-}) were from Rat MutaFlow® Kits, Litron Laboratories, and included Anticoagulant Solution, Buffer Solution, Nucleic Acid Dye Solution (contains SYTO® 13), Anti-CD59-PE, and Anti-CD61-PE. Reagents used for flow cytometric MNHEP scoring (Liver Preservation Buffer, Buffer Solution, Erythrocyte Clearing Solution, Collagenase Solution, Lysis Solution 1, Lysis Solution 2, Anti-Ki-67-eFluor® 660, DNA Stain (contains SYTOX® Green), and RNase Solution) were from Prototype *In Vivo* MicroFlow® PLUS RL Kits, Litron Laboratories. Additional supplies included Lympholyte®-Mammal cell separation reagent from CedarLane, Burlington, NC; Anti-PE MicroBeads, LS Columns, and a QuadroMACS™ Separator from Miltenyi Biotec, Bergisch Gladbach, Germany; CountBright™ Absolute Count Beads and fetal bovine serum (FBS) from Invitrogen, Carlsbad, CA; Falcon-brand 35 μm cell strainers (cat. no. 352235) from Corning, Corning, NY; and heparinized capillary tubes from Fisher Scientific, Pittsburg, PA (cat. no. 22-260-950).

2.2. Animals, treatments

Experiments were conducted with the oversight of the University of Rochester's Institutional Animal Care and Use Committee. Male Crl:CD (SD) rats were purchased from Charles River Laboratories, Wilmington, MA. Rodents were allowed to acclimate for approximately one week. Water and food were available *ad libitum* throughout the acclimation and experimental periods.

Age at the start of treatment was 6 weeks, n = 5 per group. Six-week old rats were chosen to match the JEMS/MMS group's work that has recently focused on this age [10]. DEN was prepared in water at 10x concentrations, and aliquots were frozen at −20 °C until use. On each day of administration, aliquots were thawed and added to water to prepare working solutions of 0, 0.5, 1, and 1.5 mg/mL. Administration was by oral gavage at 10 mL/kg body weight, for final dose levels of 0, 5, 10, or 15 mg/kg/day. The 15 mg/kg/day top dose was based on the report of Narumi and colleagues, who also studied male Crl:CD(SD) rats and characterized 12.5 mg/kg/day for 28 days as "about or lower than the maximum tolerated dose" [22].

2.3. Blood harvest

Day 29 blood specimens were collected into MicroFlow kit-supplied Anticoagulant Solution-coated needles and syringes *via* heart puncture exsanguination (typically 6–9 mL blood was collected per rat). For the MicroFlow assay, 50 μL aliquots of each whole blood sample were transferred to tubes containing 175 μL Anticoagulant Solution, and they were maintained at room temperature for less than 3 h until fixation with ultracold methanol as described by Torous and colleagues [23]. For *Pig-a* analyses, 80 μL of each blood sample was transferred to tubes containing 100 μL kit-supplied Anticoagulant Solution where they remained at room temperature for less than 3 h until leukodepletion as described previously [10].

2.4. Micronucleated reticulocyte assay: sample preparation, data acquisition

MN-RET and reticulocyte (RET) frequencies were determined for each of 20 blood samples *via* flow cytometry according to the *In Vivo* MicroFlow PLUS-R Kit manual, v170503 (www.litronlabs.com). These procedures have been described in detail [23,24]. MN-RET frequency measurements were based on the acquisition of approximately 20,000 high CD71-positive RET per blood sample. Instrument setup and calibration were performed using kit-supplied biological standards (*P. berghei*-infected blood cells) [25,26]. A BD FACSCalibur™ flow cytometer running CellQuest™ Pro v5.2 software was used for data acquisition and analysis.

2.5. Pig-a gene mutation assay: sample preparation, data acquisition

RET^{CD59-} and RBC^{CD59-} frequencies were determined for each blood sample *via* immunomagnetic depletion of wild-type erythrocytes and flow cytometric analysis, as described previously [13,27]. In addition to reducing analysis times to 4 min per sample, immunomagnetic depletion made it practical to evaluate many times more cells than is otherwise feasible. For instance, an average of 200×10^6 erythrocytes and 5.4×10^6 RET per sample were evaluated for the CD59-negative phenotype.

Pig-a sample labeling and washing steps utilized deep-well 96 well plates from Axygen Scientific (cat. no. P-DW-20-C) that facilitated efficient, parallel processing. Flow cytometric analyses were also conducted using 96 well plates (U-bottom, Corning, cat. no. 3799) and the BD High Throughput Sampler (HTS) provided automated, walk-away flow cytometric analysis. These variations are described in the Rat MutaFlow Instruction Manual, v140403 (www.litronlabs.com).

An Instrument Calibration Standard was created with approximately 50% wild-type and 50% mutant-mimic erythrocytes, and as described previously, it provided a means to rationally and consistently define the location of CD59-negative cells [13,27]. A BD FACSCanto II flow cytometer running Diva v6.1.2 software was used for data acquisition and analysis.

2.6. Micronucleated hepatocyte assay: sample preparation, data acquisition

Rats were anesthetized *via* CO_2 overdose, exsanguination occurred by heart puncture, and then livers were immediately excised. Wet weights were recorded, and left lateral lobes were transferred to 50 mL tubes that contained ice-cold kit-supplied Liver Preservation Buffer (includes 10% v/v dimethyl sulfoxide, added same day as use).

Approximately 1 g of each left lateral lobe was processed for flow