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Genotoxicity assessment of melamine in the *in vivo Pig-a* mutation assay and in a standard battery of assays

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ABSTRACT

The genotoxicity of melamine was evaluated with the combined *Pig-a* mutation/micronucleus assay, the bacterial reverse mutation assay, and the *in vitro* cytokinesis-block micronucleus assay (CBMN). Five groups of six- to eight-week-old male Sprague–Dawley (SD) rats were given three daily doses of vehicle control (100% pure sesame oil), melamine (500, 1000, and 2000 mg/kg) or positive control (*N*-ethyl-*N*-nitrosourea, ENU, 20 mg/kg) by oral gavage. Peripheral blood was sampled pre-dose (day −1) and at time points up to day 60. *Pig-a* mutant frequencies were determined in total red blood cells (RBCs) and reticulocytes (RETs) as RBC^{CD59-} and RET^{CD59-} frequencies, on days −1, 15, 29 and 60, and micronucleus frequencies were measured in RETs on day 4. No significant increases in RBC^{CD59-} or RET^{CD59-} frequencies were observed for the melamine-treated group at any of the time points studied, but the positive control, ENU, induced statistically significant increases compared with the vehicle control. Similar results were obtained in the micronucleus assay. Melamine did not induce statistically significant increases in %MN-RET. In the bacterial reverse mutation assay, melamine was tested from 62.5 to 1000 μg/plate in tester strains TA97a, TA98, TA100, TA102, and TA1535, with and without metabolic activation, and no evidence of toxicity or mutagenicity was observed at any dose tested. In the *in vitro* CBMN assay, in Chinese hamster ovary (CHO) cells, melamine was tested (75, 150, and 300 μg/mL) in the presence and absence of S9 mix, and no positive increases in the number of cells containing micronuclei were seen. These results suggest that melamine does not exhibit significant genotoxic potential. These data could be valuable for risk assessment purposes and also for further characterizing the new *in vivo Pig-a* gene mutation assay.

1. Introduction

During recent decades, many endpoints and test methods have been developed for evaluating the genotoxic potential of chemical agents. Those methods included *in vitro* and *in vivo* test systems. *In vitro* test systems do not replicate the metabolism and pharmacodynamics that occur *in vivo*, and this deficiency may reduce the predictive power of the results [1]. *In vivo* test systems can play more important roles in the assessment of genetic toxicity. Often, the *in vivo* test of choice is the bone marrow or peripheral blood micronucleus assay, *in vivo* chromosome aberration assay, the unscheduled DNA synthesis (UDS) assay or the *in vivo* comet assay, none of which detects gene mutations. Although a few *in vivo* gene mutation tests are mentioned in the current ICH guidelines (i.e., transgenic assays) [2], and OECD guidelines have been developed for the assays, the cost and resources associated with such studies make them less practical. Thus, a robust gene mutation assay that can be easily incorporated into repeat-dose toxicology studies would be very attractive.

Recently, a flow cytometric *Pig-a* mutation assay for measuring gene mutation in the RBCs and RETs of rats or mice has been developed [1,3–8]. These assays have advantageous characteristics and may constitute an effective tool for *in vivo* mutation and hazard assessment. For example, the *Pig-a* gene is highly conserved, a feature that facilitates studies across species of toxicological interest. Also, mutant cells accumulate with repeat dosing [9], so that even weak mutagens or exposures might be detected, and the assay is attractive for integration into repeat-dose toxicology studies. These and other strengths make the *Pig-a* assay a promising tool for regulatory safety assessments.

Melamine (2,4,6-triamino-1,3,5-triazine) is a nitrogen-containing heterocyclic triazine compound mainly used for

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the manufacture of laminates, plastics, coatings, commercial filters, glues or adhesives, and dishware, and kitchenware. There are no accepted or approved direct food uses for melamine, nor are there any recommendations in the Codex Alimentarius. Unexpectedly, this commercial industrial chemical was intentionally and illegally added to infant formula and products containing milk, leading to the melamine poisoning of pets in USA in March 2007 and a large number of infant renal calculus cases in China in September 2008 [10,11]. The potential hazards and risks caused by melamine contamination have attracted widespread attention.

Several reports have described genotoxicity test results for melamine [12,13]. However, well-designed animal studies that investigate the *in vivo* gene mutation potential of melamine are lacking. In the present study, the genotoxicity of melamine was assessed using the novel *Pig-a* gene mutation assay and a battery of short-term genetic toxicity tests recommended by ICH guidelines: the bacterial reverse mutation assay and *in vitro* and *in vivo* micronucleus assays. To reduce costs and minimize the use of experimental animals, a combined *Pig-a* mutation/micronucleus assay recommended by the International Workshop On Genotoxicity Testing (IWGT) Workgroup [14] was performed, to comprehensively assess *in vivo* genotoxicity to hematopoietic cells.

2. Materials and methods

2.1. Reagents

Melamine, sesame oil, *N*-ethyl-*N*-nitrosourea (ENU), ICR-191, 2-nitrofluorene (2-NF), methyl methanesulfonate (MMS), sodium azide, 2-aminoanthracene (2-AA), mitomycin C (MMC), cyclophosphamide (CP) and cytochalasin B (Cyto B) were purchased from Sigma–Aldrich (St. Louis., MO, USA). Anticoagulant solution, buffered salt solution, nucleic acid dye solution (contains SYTO® 13), stock anti-CD59-phycoerythrin (PE) solution, and stock anti-CD61-PE solution were from MutaFlow® PLUS-25R kits (Litron Laboratories, Rochester, NY, USA). Reagents used for flow cytometric micronucleus scoring were from MicroFlow® rat blood micronucleus kits, Litron Laboratories. Fetal calf serum (FCS), trypsin, DMEM/F12 medium, and penicillin-streptomycin solution were provided by Gibco (Grand Island, NY, USA). S9, which was prepared from male Sprague–Dawley rats induced with Aroclor1254, was from Molecular Toxicology Inc. (Boone, NC, USA). All other chemicals were of analytical grade or complied with the standards needed for cell culture experiments.

2.2. Tester strains

Five strains of *Salmonella typhimurium* (TA97a, TA98, TA100, TA102, and TA1535) were purchased from Molecular Toxicology Inc. (Boone, NC, USA), and the lot numbers were 4366D, 4367D, 4370D, 4372D, and 4369D, respectively. All strains were maintained as frozen stocks and checked for maintenance of genetic markers prior to the study.

2.3. Cell line and cell culture

The Chinese hamster ovary cells (CHO-K1) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). This cell line derives from the CHO cells originally isolated from an explant of the ovary of the Chinese hamster. The modal chromosome number of these cells is 22. The cell cycle time is 12–14 h. The cells were stored as frozen stock cultures in liquid nitrogen. Subcultures were prepared from these stocks for experimental use.

CHO-K1 cells were cultured as monolayers in DMEM/F12 medium supplemented with 100 IU/mL penicillin, 100 IU/mL streptomycin and 10% FCS. The medium was changed every 2 days, and the cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. In vivo Pig-a mutation/micronucleus assay

2.4.1. Animals, treatment, and harvests

Male Sprague–Dawley (SD) rats (age, 6–8 weeks) were purchased from Slac Laboratory Animal Company (Shanghai, China). The animals were quarantined for three days and acclimated for four days before the start of the experiments. Food and water were available *ad libitum* throughout the acclimation and experimental periods. A 12 h light/dark cycle was used and room temperature was maintained between 20 °C and 26 °C. ENU was freshly prepared each day in PBS (pH 6.0). Melamine was freshly suspended each day in 100% pure sesame oil (vehicle control). All treatments were *via* oral gavage in a volume of 10 mL/kg body weight. Five male SD rats per group were administered 0 (vehicle control, 100% pure sesame oil), 500, 1000, or 2000 mg/kg/day melamine or 20 mg/kg/day ENU (positive control) at ~24 h intervals for 3 consecutive days. Peripheral blood was collected from jugular vein on days – 1 (pre-dose), 15, 29, and 60 for *Pig-a* mutation analysis, and on day 4 for MN analysis. Approximately, 200–300 μL free-flowing blood per rat was collected directly into heparinized capillary tubes. For *Pig-a* analyses, aliquots (80 μL) of each blood sample were transferred to tubes containing 100 μL kit-supplied heparin solution where they remained at room temperature for less than 2 h until leukodepletion, as described previously. For the MN analysis, aliquots (approximately 100 μL) of each blood sample were transferred to tubes containing 350 μL kit-supplied heparin solution.

2.4.2. In vivo Pig-a gene mutation assay

As described previously, determinations of RET^{CD59-} and RBC^{CD59-} frequencies employed immunomagnetic depletion of wild-type erythrocytes prior to flow cytometric analysis [15]. In addition to reducing analysis times to approximately 4 min per sample, immunomagnetic separation made it practical to evaluate many times more cells than otherwise feasible (*e.g.*, $>150 \times 10^6$ and $>3 \times 10^6$ total erythocytes and reticulocytes per sample, respectively). An Instrument Calibration Standard (ICS) was generated on each day that data acquisition occurred. The ICS was used to optimize photomultiplier tube voltages and set fluorescence compensation. It was also used to define rationally and consistently the positions of mutant phenotype cells. A BD FACSCalibur flow cytometer running CellQuest™ Pro version 5.2.1 software was used for data acquisition and analysis.

2.4.3. In vivo micronucleus assay

Peripheral blood samples were processed for flow cytometric evaluation of micronucleated reticulocytes (MN-RET) as described in detail elsewhere [16–18]. Briefly, cells were fixed and labeled according to the instruction manual of the MicroFlow® rat blood micronucleus kit, then analyzed by a flow cytometer equipped with a 488-nm laser. Immediately before analysis, erythrocytes infected with the malaria parasite *Plasmodium berghei* (biological standard from the kit) were used to model MN-containing cells and to set-up and calibrate the instrument. The anti-CD71-FITC, anti-CD61-PE and PI fluorescence signals were detected in the FL-1, FL-2, and FL-3 channels, respectively. A total of 20,000 reticulocytes (RETs) were acquired for each sample to determine micronucleated reticulocyte frequencies (%MN-RET). The %RETs for each sample was recorded as an index of test agent toxicity to the hematopoietic system. Data were acquired on a FACSCalibur flow cytometer.

2.4.4. Calculations and statistical analyses

The formulas used to calculate mutant phenotype erythrocyte (RBC^{CD59-}) and mutant phenotype reticulocyte (RET^{CD59-})