In vivo genotoxicity assessment of acrylamide and glycidyl methacrylate

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Abstract

Acrylamide (ACR) and glycidyl methacrylate (GMA) are structurally related compounds used for making polymers with various properties. Both chemicals can be present in food either as a byproduct of processing or a constituent of packaging. We performed a comprehensive evaluation of ACR and GMA genotoxicity in Fisher 344 rats using repeated gavage administrations. Clastogenicity was measured by scoring micronucleated (MN) erythrocytes from peripheral blood, DNA damage in liver, bone marrow and kidneys was measured using the Comet assay, and gene mutation was measured using the red blood cell (RBC) and reticulocyte *Pig-a* assay. A limited histopathology evaluation was performed in order to determine levels of cytotoxicity. Doses of up to 20 mg/kg/day of ACR and up to 250 mg/kg/day of GMA were used. ACR treatment resulted in DNA damage in the liver, but not in the bone marrow. While ACR was not a clastogen, it was a weak (equivocal) mutagen in the cells of bone marrow. GMA caused DNA damage in the cells of bone marrow, liver and kidney, and induced MN reticulocytes and *Pig-a* mutant RBCs in a dose-dependent manner. Collectively, our data suggest that both compounds are in vivo genotoxins, but the genotoxicity of ACR is tissue specific.

Introduction

Acrylamide (ACR), the product of its *in vivo* metabolism, glycidamide, and glycidyl methacrylate (GMA) are structurally related: they share a vinyl group or a highly reactive oxirane (epoxy) group (Fig. 1), the presence of which triggers genotoxicity alerts [Ashby and Tennant, 1988; Tennant and Ashby, 1991]. ACR and GMA are high volume industrial chemicals employed in the manufacture of polymers and polymer coatings, finishes, adhesives, dyes, paper, and in many other applications including treating drinking and waste water. Humans may be exposed to these potential carcinogens at work or as consumers of finished goods. Also, ACR is found in cigarette smoke and in food items as a byproduct of thermal processing [Tareke et al., 2002]. GMA is a monomer in a polymerization reaction that produces a thin coating on the internal surface of metal cans intended for food storage/packaging; GMA is classified as a food contact substance by the FDA. GMA is also used in dentistry as a component of resins and adhesives [Soderholm and Mariotti, 1999]. Potentially, a large number of consumers may come into contact with and ingest non-negligible amounts of ACR and GMA on a daily basis, although the degree to which this occurs is unclear.

ACR has been studied extensively since it was identified as a human and animal neurotoxin in the late 1950s [Kuperman, 1958; McCollister et al., 1964; Garland and Patterson, 1967]. ACR is a genotoxin and a carcinogen [Mei et al., 2008; Mei et al., 2010; Wang et al., 2010; Von Tungeln et al., 2012; Beland et al., 2013]. The International Agency for Research on Cancer (IARC) classified ACR as a Group 2A compound - a probable human carcinogen, with limited evidence in humans, but sufficient evidence in animals. However, unambiguously demonstrating the *in vivo*

genotoxicity of ACR at (minimally toxic or non-toxic) doses that produce tumors remains an elusive task. In a 2-year rodent bioassay ACR was carcinogenic at doses as low as 1 mg/kg/day in mice and 2 mg/kg/day in rats; while the genotoxicity of ACR was detected at doses of 5 mg/kg/day and greater. GMA toxicity also has been studied extensively, yet the information on its in vivo genotoxic properties and carcinogenic potential remains inconclusive (Organisation for Economic Co-operation and Development (OECD) Screening Information Dataset (SIDS) report; available at <http://www.inchem.org/documents/sids/sids/106912.pdf>). GMA is not specifically mentioned in IARC publications, although other structurally related compounds, i.e., glycidol, glycidaldehyde, glycidyl stearate and glycidyl oleate (all possessing an epoxy group), are mentioned as Class 2A, 2B and Class 3 compounds.

Here we report the results of a mechanistic study of the in vivo genotoxic consequences of exposure to ACR and GMA using standardized models (i.e., the Comet assay and peripheral red blood cell (RBC) and reticulocyte (RET) micronucleus (MN) assay) as well as the novel, highly-sensitive *Pig-a* gene mutation assay. Assays were conducted in F344 rats following OECD Test Guidelines (Test No. 474: Mammalian Erythrocyte Micronucleus Test; Test No. 489: In Vivo Mammalian Alkaline Comet Assay) and consensus recommendations from the International Workshop on Genotoxicity Testing [Gollapudi et al., 2015].

Materials and Methods.

Animals, chemicals and treatments. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the National Center for Toxicological Research.

Solid ACR was of molecular biology gel electrophoresis grade from Bio-Rad (Hercules, CA). A 40% (w/v) stock solution of ACR was made with tissue culture grade water, filter-sterilized and stored at 4°C. Groups of six 8-week-old Fisher 344 male rats were gavaged with freshly made working stocks of ACR to achieve doses of 0.33, 0.66, 1.32, 2.7, 5, 10, and 20 mg/kg/day continuously for up to 29 days (see Fig. 2).

Liquid GMA was purchased from Aldrich (Sigma-Aldrich, St. Louis, MO; cat. no.: 151238, 97% pure) and stored at 4°C. The commercial GMA contained 100 ppm of an inhibitor, monomethyl ether hydroquinone, and 0.02% of epichlorohydrin impurity (Fig. 1). For the *Pig-a*, MN and Comet assays, groups of six male rats were gavaged for up to 29 days with freshly made stocks of 10, 20 and 30 mg/ml GMA in sesame oil to achieve doses of 0, 50, 100 and 150 mg/kg/day (Fig. 2). GMA dosing started at 8.5 weeks of age. In addition, for the short-term Comet assay a separate group of 3 male rats was treated with a dose of 250 mg/kg/day GMA (using a 50 mg/ml stock) for 3 days starting at ~12 weeks of age.

For the Comet assay, an additional 4 to 6 male rats were used as positive controls.

These animals were gavaged with freshly prepared 10 mg/ml methyl methanesulfonate (MMS; Sigma-Aldrich) in PBS 3 hours prior to sacrifice. The final dose of MMS was 100 mg/kg.

The first day of the treatment was designated as Day 1. Blood collections for the MN test and the RBC *Pig-a* assay occurred via tail vein on the days shown in Fig. 2.

The MN assay. The frequency of MN peripheral blood reticulocytes was determined using the Rat MicroFlow kit (Litron Laboratories; Rochester, NY) on Day 4 of the treatment (after 4 administrations of the test articles) and on Day 29 (ACR experiment only). Instructions provided by the kit manufacturer were followed, with the exception that reaction volumes were scaled down 50%. The samples were processed on a FACSCanto II flow cytometer (Becton-Dickinson; Milpitas, CA) and frequencies of MN RETs were calculated as suggested by the manufacturer of the MicroFlow kit. The kit included a scoring control that was used as a positive control.

The *Pig-a* assay. The *Pig-a* assays were performed before starting treatment and on Days 15, 29, and 56 using the Rat MutaFlow kit (Litron). Blood (~150 µl) for the *Pig-a* assays was drawn from the tail vein using a 1-ml syringe equipped with a 25-Ga needle; immediately, the blood was transferred from the syringe to a 1.5-ml microcentrifuge tube and exactly 100 µl was mixed in another microcentrifuge tube with 100 µl of anticoagulant solution from the kit. Blood labeling, magnetic depletion and analyses were consistent with the suggestions from the manufacturer of the MutaFlow kit. The samples were processed on a FACSCanto II flow cytometer and the *Pig-a* mutant frequencies (MFs) were calculated according to the instructions provided by the kit manufacturer. Mutant-mimic samples (prepared according to manufacturer's instructions), rather than positive control animals, were used as a positive control for the *Pig-a* assay.

The Comet assay. Rats were euthanized 3 hours after the final treatment. Liver and kidney cells were released by mincing the tissues in Ca^{2+} , Mg^{2+} -free HBSS supplemented with 20 mM EDTA and 10% DMSO (mincing buffer). Then the cell suspension was filtered through a 40- μm cell strainer (Fisher Scientific; Pittsburg, PA) for removing tissue chunks. Bone marrow cells were isolated by flushing femurs with mincing buffer.

The alkaline Comet assay was performed according to the protocol endorsed by the Japanese Committee for the Validation of Alternative Methods (currently OECD Test No. 489: In Vivo Mammalian Alkaline Comet Assay). Briefly, single cell suspensions were mixed with 0.5% NuSieve GTG low melting-point (LMP) agarose (Lonza; Rockland, ME) at 37°C at a 1:10 (v:v) ratio. 100 μl of these mixtures were applied onto glass microscope slides pre-coated with 1% agarose. After solidification of the LMP agarose, the slides were immersed in freshly prepared lysis buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, with 10% DMSO and 1% Triton X-100 added just before use) and stored at 4°C in the dark overnight. The slides then were transferred into a chilled alkaline solution (300 mM NaOH, 1 mM EDTA, pH >13). After DNA unwinding for 20 min, electrophoresis was performed in the same solution at 4°C in the dark for 20 min at 0.7 V/cm. After washing with neutralizing buffer (0.4 M Tris, adjusted to pH 7.5 with HCl) 3 times for 5 min each wash, the slides were fixed in ice cold 100% ethanol and dried overnight.

Prior to scoring, the slides were stained with SYBR Gold (Invitrogen, Carlsbad CA) (1:10,000 dilution in 1 $\times$ Tris/Borate/EDTA buffer, pH8). Two slides were scored from each collected tissue; 75 cells were selected randomly from each slide and scored

using a system consisting of a Nikon 50i fluorescent microscope equipped with a Plan 20× objective, a 534/20 nm excitation and 572/28 nm emission SpGold filter set (Semrock; Rochester, NY), and Comet IV digital imaging software (Perceptive Instruments, Bury St Edmunds, UK). Percent (%) DNA in tail, defined as the fraction of DNA in the tail divided by the total amount of DNA associated with the cell multiplied by 100, was used as the parameter for assessing DNA damage. To avoid bias, the Comet slides were scored by an independent operator unfamiliar with the details of the experimental treatment design.

Histopathological evaluations. Tissues collected from the animals designated for the Comet assay were preserved in 10% neutral buffered formalin, imbedded in paraffin, sectioned at 5 μ m, stained with hematoxylin and eosin, and examined by microscopy by Toxicologic Pathology Associates (TPA; Jefferson, AR). TPA also performed gross evaluations of tissues at the time of necropsy.

Statistical analyses. SigmaPlot for Windows v.11 (Systat Software, San Jose, CA) was used for data processing. One way ANOVA was used for comparing multiple treatment groups at specific time-points (for processing data generated in the MN, *Pig-a* and Comet assays); and one way repeated-measures ANOVA was used for detecting changes within the same treatment group over a period of several time-points (for processing *Pig-a* data only). If raw data were not normally distributed (as determined by SigmaPlot), ANOVA on ranks was performed. P-values of less than 0.05 indicated significant differences between the groups. In such cases the Tukey test was used for pairwise comparisons of data for different treatment groups or for different time-points within the same group. A T-test was used only for comparing Comet assay data in

animals treated with 250 mg/kg/day for 3 days and in the concurrent vehicle-control group. Statistical analyses of the Comet assay data were performed on median and mean values of percent DNA in tail.

Results.

General observations. ACR treatment was uneventful with the exception of a few animals from the 20 mg/kg/day treatment group that exhibited mild signs of paresis of the hind limbs by Day 28 of treatment. The signs of paresis disappeared after the treatment was discontinued. All rats treated with the various doses of ACR gained weight at the same rate as vehicle-gavaged rats.

There were no gross observations reported at necropsy or during the tissue preparation phase. Upon microscopic examination of liver slides there were no findings attributable specifically to the administration of ACR. Minimal severity multifocal mononuclear cell infiltration, infrequent necrotic hepatocytes or a small amount of cellular debris were observed in the livers of all animals, vehicle-control and ACR-treated, and were not related to the treatment with ACR.

Before initiating the main GMA study, a small scale dose range-finding study was performed with doses of 40, 70, 100, 150, 200, 250, 300, 350, and 400 mg/kg/day using only 2 animals per dose. The dosing with 200, 250, 300, 350 and 400 mg/kg/day GMA was discontinued after Days 15, 3, 2, 2 and 1, respectively, due to sudden loss of weight or mortality. Doses up to 150 mg/kg/day GMA were tolerated by the animals for 28 days. Animals for which the treatment was discontinued re-gained weight normally. In the main GMA study, with 6 animals per group, a reduction in weight gain in treated groups was notable – over the 28-Day treatment schedule the high-dose-treated rats gained only half as much weight as the vehicle-control group (Fig. 3).

A dosing error was made on Day 8 of treatment with GMA – three animals from the control group designated for the *Pig-a* assay were accidentally gavaged once with

150 mg/kg of GMA. The accidental treatment with a single dose of GMA did not noticeably affect the three rats as determined by the pattern of weight gain and the subsequent *Pig-a* assays.

In 5 of the 6 rats treated with 150 mg/kg/day GMA for 29 days a gross pathology (mass and nodules) in the forestomach was noted at the time of necropsy and upon microscopic examination described as squamous hyperplasia. A gross “lesion” (having the microscopic correlate of necrosis) was observed in one of three rats treated with 250 mg/kg/day GMA for 3 days. Non-neoplastic squamous hyperplasia was confirmed microscopically in GMA-treated rats with increasing numbers of affected animals and severity in groups treated with the higher GMA doses (e.g., forestomach hyperplasia in 5 out of 6 rats in the 150 mg/kg/day×29 group and in 3 out of 3 rats in the 250 mg/kg/day×3 group); in addition, forestomach edema was confirmed in 3 out of 3 rats in the 250 mg/kg/day×3 group. These observations were consistent with the route of delivery of GMA, gavage. These lesions were not observed in vehicle-control animals. No gross observations were recorded in the liver or kidney of GMA-treated animals. The kidneys and the livers from GMA-treated animals (gross and microscopic) were consistent with the normal phenotype observed in the vehicle-control group.

Genotoxicity assessments. Clastogenicity/aneugenicity was measured by induction of MN RETs in peripheral blood. ACR was negative with the chosen treatment regimen, both on Day 4 of continuous daily treatments, and on Day 29 (after 28 consecutive treatments) of the study (Fig. 4A).

GMA was positive on Day 4 of the study; the frequency of MN RETs increased in a dose-related manner in treated animals, with the increase in the group treated with 150 mg/kg/day being statistically significant (Fig. 4B).

Mutagenicity was measured by the induction of CD59-deficient *Pig-a* mutant RBCs and RETs in peripheral blood. The only evidence of ACR mutagenicity was observed in rats treated for 28 days with 20 mg/kg/day and assays on Day 56 of the study, i.e., 28 days after the last treatment (Fig. 5A). No collaborating increases in the frequency of CD59-deficient RETs were observed at any time-point (Fig. 5B). One animal in the control group produced unexpectedly high values for RBC and RET *Pig-a* MFs on Day 56. But even if this outlier was excluded from statistical analyses, the overall results remained the same.

On the other hand, evidence for mutagenicity of GMA was observed in the total RBC fraction of rats treated with 50, 100 and 150 mg/kg/day on Days 29 and 56 of the study using repeated-measures statistical assessment (Fig. 5C). In addition, the increase in *Pig-a* mutant total RBCs was corroborated with the increases in the frequency of mutant RETs for the 100 and 150 mg/kg/day doses and at time-points as early as Day 15 (Fig. 5D).

DNA damage was measured using the alkaline Comet assay. The tissues for the Comet assay were chosen based upon previously published data on in vivo genotoxicity for ACR [Rothfuss et al., 2010] and on GMA general toxicity (OECD SIDS report). Analyses of the Comet assay data using median and mean values of percent DNA in tail resulted in similar statistical outcomes.

Dosing with 10 mg/kg/day ACR for 29 consecutive days resulted in DNA damage in the liver (quantified as percent tail DNA; Fig. 6A). Considering the historical background levels of DNA damage in liver cells of untreated animals in our laboratory of 2.7 ± 0.9 (average $\pm$ SD; for 32 animals), such an increase may be regarded as weak. No increase in DNA damage was detected in bone marrow cells from ACR-treated rats.

DNA damage was detected in all tested tissues from animals treated with GMA. DNA damage was increased in bone marrow and liver cells of rats treated with one or more concentrations of GMA for 29 days (Fig. 6B), and in bone marrow, liver and kidney cells of animals treated with a higher dose of GMA, 250 mg/kg/day, but for a shorter period of 3 days (Fig.6C).

In the Comet assay, the levels of DNA damage in tissues of positive control animals treated with MMS were consistent with historical data established in our lab: 10-fold higher than in vehicle-control animals for cells from liver and bone marrow, and 2-fold higher than in vehicle-control animals for cells from the kidney.

Discussion

ACR and GMA are high-volume industrial chemicals that are not used directly at the consumer level. Although known as being hazardous materials, manufacturing of these chemicals is controlled so that only small populations can be accidentally exposed to them (e.g., employees in their work environment). The revitalized concern about these chemicals is that they may be present in food and subsequently may affect much wider populations. ACR was found at concentrations of up to 3.5 mg/kg in thermally treated (fried, grilled or baked) starchy items [Tareke et al., 2002]. GMA is used to make polymeric materials that the FDA and EFSA have authorized for use in food packaging, and it is conceivable that the GMA monomer may leach into food from such packaging.

We undertook a mechanistic study for detecting the genotoxicity of ACR and GMA applying standard or recommended tests for nonclinical safety evaluations of drugs intended for human use. The detection of in vivo MN RBCs and RETs was used as a test for clastogenicity and aneugenicity. The *Pig-a* assay was used as a test for mutagenicity; and the Comet assay was used as a test for DNA damage. The overall experiment was designed to mimic integration of genotoxicity tests into standard 28-day repeated dose general toxicology evaluations with a dosing range up to maximum tolerated dose (OECD Test No. 407: Repeated dose 28-day oral toxicity study in rodents).

ACR doses for this study were chosen from the available literature on genotoxicity and carcinogenesis [Rothfuss et al., 2010; Beland et al., 2013]. GMA doses for the main study were chosen from a preliminary scale dose range-finding experiment. The reported oral acute LD₅₀ dose of GMA is about 600 mg/kg; surprisingly, a dose of

150 mg/kg, which is 25% of the acute LD₅₀, was tolerated by rats for 29 consecutive days. The body of literature on ACR and GMA suggests that both should be weak genotoxins. For this reason the study was organized to maximize the sensitivity of each employed test. The maximum response in the *Pig-a* assay (a test that is capable of detecting the response to cumulative treatment dose) is achieved in the RET fraction about 2 weeks after the last treatment and in the RBC fraction about 8 weeks after the last treatment, yet the response in the Comet and MN assays decreases sharply within days after cessation of the treatment. The Comet assay requires termination of animals, while the MN and *Pig-a* assays require only small amounts of peripheral blood. As such, we employed two cohorts of experimental animals: one for the *Pig-a* and MN assays (in which both assays were performed on the same set of animals, with blood collections on Days -1, 4, 15, 29 and 56), and another for the Comet assay (these animals were terminated for sampling after 3 or 29 daily treatments).

Previously it was shown that doses of 5 mg/kg/day ACR and higher induce robust responses in the Comet assay in a 29-day treatment protocol [Rothfuss et al., 2010]. Although we couldn't demonstrate a statistically significant response for the 5 mg/kg/day dose, the 10 mg/kg/day dose produced a 2.8-fold increase in DNA damage, but only in liver cells. It might be relevant that the positive response determined previously using an ACR dose of 5 mg/kg/day employed a different timing between the last treatment and tissue collection, 2 hours (instead of the 3-hour interval in our experiment). Such a 1-hour difference in timing may affect the measurement of DNA damage in the Comet assay considering the rapid elimination kinetics of ACR in the rat [Rothfuss et al., 2010]. It is unlikely that the low levels of inflammation in the liver noted

in the histopathological examination affected the outcome of the Comet assay in our experiment - inflammation was present at similar levels in control and treated rats.

The lack of response in the Comet assay in bone marrow cells in our study is consistent with the outcomes of the MN and *Pig-a* assays. Usually, the MN and *Pig-a* assays reflect genotoxic activity in bone marrow – if there is no DNA damage in bone marrow, the chances of forming MN or mutations are low. Overall our observations are in accord with the findings of the collaborative study in which ACR did not increase the frequency of MN RETs at any dose tested [Rothfuss et al., 2010]. Also, hematopoietic malignancies are not characteristic of ACR-treated animals. Based on this information, ACR can be characterized, at best, as a weak-to-modest tissue-specific genotoxin and carcinogen in rats.

According to reports, GMA can form DNA adducts [Fang et al., 1999], and it is genotoxic in vitro [Canter et al., 1986; von der Hude et al., 1991]. In our study, GMA was genotoxic in rats in all the assays that were employed and in all tissues tested (bone marrow, liver and kidney). In the 28-day study, the response to GMA was the most robust and dose-dependent in the liver. Kidney was chosen for examination in our study based on a previous report that absolute and relative kidney weights were increased in animals treated with 100 mg/kg/day GMA for 45 days (OECD SIDS report). We didn't observe kidney weight increases, but we saw a significant increase in DNA damage in kidney cells in the 3-day treatment with a toxic dose of 250 mg/kg/day GMA. The increase in DNA damage in bone marrow was accompanied by an increased frequency of MN RETs in peripheral blood on Day 4 and increases in the frequencies of *Pig-a* mutant RBCs and RETs. While performing the *Pig-a* assay, concurrent increases

in the frequencies of mutant RBCs and RETs are important for reliably classifying a positive response [Gollapudi et al., 2015], The most dramatic effect of the treatment with GMA was observed in the forestomach, which is the immediate point of contact. This result is consistent with findings mentioned in the OECD SIDS report. It is possible that DNA damage in the cells of forestomach is even higher than in the liver. Unfortunately, performing the Comet assay on forestomach cells is challenging due to difficulties in processing which may contribute to extremely high backgrounds. Based on our results, GMA can be characterized as a systemic genotoxin and mutagen in rats.

We have used an approach to statistical analysis of data generated with the *Pig-a* assay that is different from the one used so far in *Pig-a* studies. Conventionally, the *Pig-a* MF data determined in treatment and control groups (composed of different subjects) are compared, with separate pair-wise comparisons conducted for data obtained at each specific sampling time-point (IWGT Workgroup recommendation [Gollapudi et al., 2015]). At the same time, it is a common practice to acquire *Pig-a* data before the initiation of the treatment (e.g., in order to identify and eliminate potential outlier animals before committing to a long-term repeated administration study) and at several time-points after that (while on or after the treatment) in the same animals. Assuming that 1) the antibody/stain labeling and magnetic enrichment protocols are reproducible, 2) the flow cytometer used for collecting data has no appreciable drift in measuring fluorescence signal, and 3) the same gating strategy is used for analysis of all samples and at all time-points, the comparisons of *Pig-a* MFs can be determined within the same treatment group before, during and after the treatment. Such comparisons may be more sensitive because the values collected prior to treatment can

be used as individual controls for each experimental animal [Glantz, 1992]. The mathematical apparatus for such comparisons (e.g., paired T-test or repeated-measures ANOVA) is available in most statistical software packages. Longitudinal analyses may not require a separate vehicle-control group and, theoretically, can be useful (in certain situations) for reducing the total number of experimental animals. In our study, using the “before-during-after” longitudinal approach for analyses of *Pig-a* MF data was essential as an error in dosing of three vehicle-control animals practically invalidated the whole vehicle-control group. Nevertheless, we also performed the standard comparisons of the control and treatment groups as if no error of dosing of the control occurred, as we believe that GMA, being a relatively weak mutagen, may not induce a detectable response after a single administration. Our results suggest that repeated-measures ANOVA can identify statistically significant *Pig-a* responses to GMA treatment earlier and at lower treatment doses than the standard control vs. treatment groups ANOVA.

In conclusion, using a novel combination of genotoxicity assays we have characterized GMA as a systemic in vivo genotoxin. It remains to be determined what risk it may represent to consumers of food that potentially contain trace amounts of GMA, but our results warrant future studies of the potential impact of GMA on human health. Another unanswered question may be to what degree a genotoxic contaminant present in commercial GMA stocks, epichlorohydrin [Rossi et al., 1983], contributed to the effect determined in this study or may contribute to potential genotoxicity in consumer products.

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Contributions

VND – study design, in vivo work, flow cytometry/*Pig-a* assay, drafting the manuscript.

MMPM – flow cytometry.

LPM – in vivo work.

MGP – in vivo work, flow cytometry/*Pig-a* assay.

WD – the Comet assay.

Figure legends

Figure 1. Chemical structures. Acrylamide is believed to be metabolized into glycidamide by cytochrome P450-containing mixed function cellular oxidases. Glycidamide and glycidyl methacrylate (GMA) have an epoxy (oxirane) group (alternative name of GMA is 2,3-epoxypropyl-acrylic acid ester). The epoxy group is highly strained; it can react with proteins and DNA, and form pro-mutagenic DNA adducts. Epichlorohydrin is an unreacted contaminant in commercial preparations of GMA. GMA is produced by reacting methacrylic acid with epichlorohydrin.

Figure 2. Schedules for treatment of Fisher 344 male rats with acrylamide (ACR) and glycidyl methacrylate (GMA) by gavage and relative timing for performing various genotoxicity analyses. In the GMA experiment an accidental error in dosing occurred on Day 8 (marked with *): three animals in the vehicle-control group were gavaged once with 150 mg/kg dose of GMA instead of the vehicle, sesame oil.

Figure 3. Weight gain in male Fisher 344 rats gavaged with glycidyl methacrylate (GMA) for 28 days. Rats gavaged with 100 and 150 mg/kg/day gained significantly less weight than the control animals (one way ANOVA; marked with *). Animals designated for the MN/*Pig-a* and the Comet parts of the study were weighed (total of 12 animals for each GMA treatment group). Error bars represent standard deviation.

Figure 4. The frequency of micronucleated (MN) reticulocytes (RETs) in rats treated with acrylamide (ACR) or glycidyl methacrylate (GMA). A. No significant effect of

treatment with ACR was observed at Days 4 and 29. B. In rats treated with GMA, a dose-related increase in the frequency of MN RETs was observed on Day 4, with the response in high dose-treated rats vs. control rats being statistically significant (one way ANOVA with Tukey test; shown with *). Error bars represent standard deviation.

Figure 5. *Pig-a* data statistical analyses. Average total RBC (A and C) and reticulocyte (RET; B and D) *Pig-a* MFs are shown for animals treated with acrylamide (ACR; A and B) or glycidyl methacrylate (GMA; C and D). Standard deviation error bars are not shown in order to avoid cluttering. Complete *Pig-a* data for each experimental animal at each time-point can be found in Supplemental Table I. Data related to different time-points are shown by a different color (or a different shade of gray). *Pig-a* MFs for different treatment groups at any specific time-point (before treatment (Day -1) and Days 15, 29 and 56) were compared by one-way ANOVA (e.g., eight groups treated with the range of ACR doses from 0 to 20 mg/kg/day at Day 56). Statistically significant differences between the treatment groups are indicated by * (marked for the group identified in Tukey test). In the ACR experiment, only the Day 56 high dose-treated group (20 mg/kg/day) showed a statistically significant elevated *Pig-a* MF; even then it was different not from the vehicle-control group, but rather from the group treated with the low dose ACR (0.33 mg/kg/day). Responses to treatment were compared for the animals in each treatment group over several time-points (e.g., the 20 mg/kg/day ACR group at Days -1, 15, 29, and 56) using one way Repeated Measures ANOVA. A significantly different response to treatment at a specific time-point (compared to Day -1 data, as determined by Tukey test) is indicated by an open triangle ($\triangle$) under the

appropriate bar. The *Pig-a* data for the control animals in the GMA experiment for Days 15, 29 and 56 are shown either as combined (solid light gray, dark gray and black color bars) or separated into two subgroups shown with the left or right hatched color pattern bars representing the three rats mistakenly (m) treated once (on Day 8) with the high dose of GMA and the remaining (r) true vehicle-control rats. The blue bars represent *Pig-a* data determined on Day 29 for the six vehicle-control rats designated for the Comet assay (c); it is shown for comparison purposes only, although it can be used as a vehicle-control group for comparing the response to GMA treatment measured at Day 29. Overall using longitudinal data generated for each treatment group (and each individual animal) for statistical evaluation may provide a more sensitive tool for detecting the effect (the induction of *Pig-a* mutant RBCs and RETs) of treatment with weak mutagens.

Figure 6. The Comet assay with tissues from F344 male rats treated repeatedly with acrylamide (ACR) or glycidyl methacrylate (GMA). Data are shown as mean %DNA in tail for samples of bone marrow, liver and kidney cells. Note the difference in scale of vertical axes in the ACR and GMA experiments. A: a weak but statistically significant increase of DNA in tail was noted in the liver of animals treated with 10 mg/kg/day ACR, the highest ACR dose tested in the Comet assay. B: in animals treated with 29 consecutive doses of up to 150 mg/kg/day GMA statistically significant increases of DNA damage were determined for bone marrow cells for a GMA dose of 100 mg/kg/day and liver cells for GMA doses of 100 and 150 mg/kg/day (one way ANOVA followed by Tukey test; marked with *). C: using a truncated treatment schedule and a higher GMA

dose of 250 mg/kg/day for 3 days, significant increases in DNA damage were observed in bone marrow, liver and kidney (Student's T-test); the 250 mg/kg/day dose was toxic in the preliminary 28-day dose range-finding study. Error bars represent standard deviation.

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Figure 1

Acrylamide

Glycidamide

Glycidyl methacrylate

Epichlorohydrin

Figure 2

ACR experiment

MN and Pig-a schedule for dosing with 0, 0.33, 0.66, 1.32, 2.7, 5, 10, and 20 mg/kg/day (6 rats in each group)

Day	-1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	56
Procedure	b	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	b	b
Assay					M											P														P	P

Comet assay schedule for dosing with 0, 1.32, 2.7, 5, and 10 mg/kg/day (6 rats in each group)

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	
Procedure	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	x
Assay																														C

Comet assay schedule for single dosing with positive control, 100 mg/kg MMS (6 rats)

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Procedure																												g	x
Assay																													C

GMA experiment

MN and Pig-a schedule for dosing with 0, 50, 100 and 150 mg/kg/day (6 rats in each group)

Day	-1	1	2	3	4	5	6	7	8*	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	56
Procedure	b	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	b	b
Assay					M											P														P	P

Comet assay schedule for dosing with 0, 50, 100 and 150 mg/kg/day (6 rats in each group)

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29		
Procedure	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	g	b	x
Assay																															<div>C</div> <div>P</div>

Comet assay schedule for high dose 250 mg/kg/day GMA and concurrent vehicle-control (3 rats in each group)

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	
Procedure																											g	g	g	g
Assay																														C

Comet assay schedule for single dosing with positive control, 100 mg/kg MMS (4 rats)

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Procedure																												g	x
Assay																													C

b blood collection

x tissue collection

g gavage

P The Pig-a assay

C The Comet assay

M The Micronucleus assay

Figure 3

Figure 4

A. ACR experiment

B. GMA experiment

Figure 5

ACR experiment

A.

B.

GMA experiment

C.

D.

Figure 6

A. ACR experiment,
29 consecutive treatments

B. GMA experiment,
29 consecutive treatments

C. GMA experiment,
3 consecutive treatments