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Induction of micronuclei in mouse and rat by glycidamide, genotoxic metabolite of acrylamide

Birgit Paulsson [a], Natalia Kotova [b,c], Jan Grawé [b,1], Alistair Henderson [d], Fredrik Granath [e], Bernard Golding [d], Margareta Törnqvist [a,*]

[a] *Department of Environmental Chemistry, Stockholm University, S-10691 Stockholm, Sweden*
[b] *Department of Genetic and Cellular Toxicology, Stockholm University, S-10691 Stockholm, Sweden*
[c] *Department of Radiobiology and Environmental Medicine, International Sakharov Environmental University, Minsk, Belarus*
[d] *Department of Chemistry, University of Newcastle, Bedson Building, Newcastle NE17RU, UK*
[e] *Department of Medical Epidemiology, Karolinska Institutet, S-17177 Stockholm, Sweden*

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Abstract

Male CBA mice and male Sprague–Dawley rats were treated by i.p. injection of glycidamide (GA), the presumed genotoxic metabolite of acrylamide (AA). GA was obtained through a new way of synthesis. As an endpoint of chromosome damage, micronucleus (MN) induction in erythrocytes was measured. Hemoglobin (Hb) adducts were used as a measure of in vivo dose of GA. GA induced linear dose-dependent increases in adduct levels in both species. Rats exhibit, compared with mice, 30% higher Hb adduct levels per unit of administered amount of GA. The incremental MN frequencies per administered dose of GA in mice showed a linear-quadratic dose-dependent curve. In the rat no positive dose–response relationship was obtained, probably due to toxic effects to the bone marrow. The main result of this study is the finding that after treatment with synthetic GA the MN frequency per unit of the in vivo dose of GA in the mouse is very similar to that obtained in a previous study, where animals were treated with AA and GA as a metabolite. This equality in potency of GA, whether its in vivo dose is established by injection of synthetic GA or through metabolism of AA, supports the view that GA is the predominant genotoxic factor in AA exposure.

Keywords: Glycidamide; Acrylamide; Micronuclei; Hemoglobin adducts; Carcinogenic

1. Introduction

Acrylamide (AA) is a vinylic compound used as monomer in the synthesis of polyAA products for, e.g. grouting and soil stabilization. AA is neurotoxic and clastogenic and is known to be carcinogenic in animal experiments and, therefore, classified as a probable human carcinogen [1]. Glycidamide (GA) is a metabolite of AA formed through metabolic transformation [1]. AA is metabolized to GA by cytochrome P450 enzymes (Fig. 1), as shown by Sumner et al. [2], by comparing metabolism of AA in wild-type mice, mice pretreated with a P450 inhibitor and mice devoid of cytochrome P450 2E1. AA has shown low reactivity towards DNA in vitro [3] and in AA-treated mice and rats only DNA adducts from the metabolite GA

* Corresponding author. Tel.: +46-8-163769; fax: +46-8-152561.
E-mail address: margareta.tornqvist@mk.su.se (M. Törnqvist).
[1] Present address: The Cell Analysis Core Facility, The Rudbeck Laboratory, Uppsala University, S-75105 Uppsala, Sweden.

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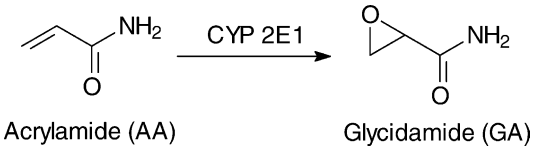

Fig. 1. AA metabolized in vivo (by CYP 2E1 enzymes) forming GA.

have been detected [4]. Therefore, GA is assumed to be the genotoxic agent in AA exposure [4,5].

In cancer tests mice are more sensitive with regard to tumor induction per unit of absorbed lifetime dose of AA, compared to rats [1,6]. Furthermore, the mouse is more effective than the rat in metabolizing AA to GA, as shown by Sumner et al. [7] analyzing urinary metabolites from rats and mice following AA administration, and by Paulsson et al. [8] measuring hemoglobin (Hb) adducts from AA and GA in mice and rats after AA treatment. These observations were taken by Paulsson et al. as a support of the applicability of the multiplicative cancer risk model for AA [5]. This model implies that the increase in cancer risk of a genotoxic chemical is proportional to the in vivo dose of the genotoxic compound, in this case GA, and the background tumor frequency [9].

This study is part of a project aiming at an improved cancer risk assessment of AA. The study is focused on the genotoxicity of GA per dose unit in mice and rat. The level of Hb adducts of GA is used as a measure of in vivo dose, while micronucleus (MN) frequencies in erythrocytes are used as a measure of chromosome damage. In a previous study mice and rats were treated with AA, MN frequencies were measured and Hb adduct levels were determined from AA and the metabolite GA. MN induction was, in that study, observed only in mouse. This was suggested to be a result of the more effective metabolic conversion of AA to GA in the mouse, compared with the rat, leading to higher in vivo doses of GA per unit of administered dose of AA in the mouse [8].

The objectives of the present study were to study the genotoxic potency per in vivo dose of GA, for further elucidation of the role of GA in the genotoxic and carcinogenic effect of AA. The genotoxic effects were studied using a flow cytometric in vivo MN assay, measuring the frequencies of micronucleated polychromatic erythrocytes (MPCE) in mouse peripheral blood and in rat bone marrow. In vivo GA doses were measured as levels of GA adducts to N-terminal valine in Hb. From a measured level of a specific, stable adduct to a macromolecule with known turnover kinetics the in vivo dose can be calculated. The in vivo dose implicates the net effects of all enzymatic, chemical and physiological factors on rates of formation and disappearance of a reactive chemical or metabolite. The in vivo dose is, if defined as the concentration in tissues integrated over time [10], proportional to the frequencies of mutations [11].

2. Materials and methods

2.1. Laboratory animals

Male CBA mice, 8 weeks old, weighing about 25 g, and male Sprague–Dawley rats, 8 weeks old and weighing about 300 g, were used. The mice were supplied from B&K Universal AB, Sollentuna, Sweden, and the rats from Charles River Sverige AB, Uppsala, Sweden. The animals were kept in the animal house at Stockholm University, where they were allowed free access to solid food and tap water. The study has been reviewed and approved by the Ethical Committees on Animal Experiments of Stockholm, Sweden.

2.2. Chemicals

AA (CAS no. 79-06-1) was obtained from Merck (acrylamide for electrophoresis, 99%), oxone (potassium monopersulfate) (CAS no. 37222-66-5) was obtained from Sigma–Aldrich and pentafluorophenyl isothiocyanate (CAS no. 35923-79-6) was obtained from Fluka (purum, >96%, purified on a Sep-Pak silica cartridge [12]). All other chemicals and solvents used were of analytical grade.

GA (CAS no. 5694-00-8) was synthesized through reaction between AA and dimethyldioxirane (DMD) (Fig. 2). Firstly, DMD was synthesized by separately adding oxone (200 g) and water/acetone (500 ml of 420/320 ml mixture) to a vigorously stirred solution of $NaHCO_3$ (100 g) in water/acetone (240 ml of 420/320 ml mixture) over a 3 h period, at a reduced air pressure (730 mmHg) [13]. DMD in acetone was collected over a 2.5 h period. After titration of the distillate, AA (1 mol eq.) was added to DMD in

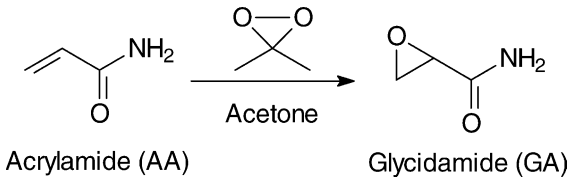

Fig. 2. Racemic GA was synthesized through reaction between AA and dimethyldioxirane in acetone.

acetone (1.5 mol eq.) for synthesis of GA. The solution was stirred under N_2 at ambient temperature for 3 days. The acetone was removed and the product was purified by distillation. The purity of the GA was confirmed by NMR analysis: ^{1}H NMR (d_6-DMSO) 2.75 (1H, dd, *J* 6.3 and 2.4 Hz, CH), 2.89 (1H, dd, *J* 6.3 and 4.5 Hz, CH), 3.29 (1H, dd, *J* 4.3 and 2.6 Hz, CH), 7.34 (1H, b, s, NH) 7.49 (1H, b, s, NH).

2.3. Animal treatment

GA was dissolved in 0.9% saline under sterile conditions prior to the treatment of the animals. All treatments were done by single i.p. injection. The injection volume was for mice 10 μl/g, and for rats 8 μl/g. A pilot study was performed where two animals per species, treated with 0.7 mmol/kg body weight, and one control animal per species, were analyzed by the MN assay (see below). Both species showed, at this administered dose, a decrease in the percentage of PCE, an indication of toxic effects to the bone marrow. The decrease was more pronounced in the mice (∼40%) than in the rats (∼20%) (see also Section 3). Therefore, it was decided that in the main study, 0.7 mmol/kg body weight should be the highest administered dose to mice. For the rats, however, the highest administered dose was set to 1.4 mmol/kg body weight, with expectations of obtaining higher MN frequencies.

In the main study three animals per administered dose and four controls of each species were used. The mice were treated with three doses (0.18, 0.35, and 0.70 mmol/kg body weight). For the rats only two different doses (0.70 and 1.4 mmol/kg body weight) were used, in order to minimize the need for animals and for GA. The last rat treated, scheduled for 1.4 mmol/kg body weight, was given only 0.90 mmol/kg body weight due to insufficient amount of substance available.

For mice, peripheral blood PCE were studied for MN induction, while for rats bone marrow PCE were used. Peripheral blood is better suited for low-dose studies due to a lower degree of variability, but in rats the spleen selectively removes micronucleated PCE. However, we have earlier shown, for both mice and rats, that within species MN frequencies measured in peripheral blood and bone marrow are quantitatively comparable, providing that samples are taken at the respective optimal times for each tissue [14,15]. In this study, rat bone marrow was sampled 24 h after treatment, and mouse peripheral blood 48 h after treatment, based on previous studies where these times showed maximum responses for the respective tissues [8]. The animals were weighed before and after treatment.

2.4. Hb adduct analysis by the N-alkyl Edman method

One of the major reactive sites in Hb of human and rodents is the N-terminal valines, and many compounds, for instance AA and GA [16] can be monitored in vivo, through their adducts to the N-termini by the *N*-alkyl Edman method [17,18]. After derivatization with the Edman reagent, pentafluorophenyl isothiocyanate (PFPITC), the adduct (the *N*-substituted N-terminal valine) is detached from the globin as an Edman derivative, the *N*-alkylvaline-pentafluorophenylthiohydantoin (alkyl-Val-PFPTH). The derivative is then analyzed by gas chromatography–mass spectrometry (GC–MS).

Red blood cells were separated from the plasma and the globin precipitated [19]. Samples of globin were prepared according to Törnqvist et al. [20] with modifications according to Bergmark [21] and analyzed by GC–MS/MS after acetonization of the GA-Val-PFPTH derivative, according to Paulsson et al. [8] and Paulsson et al. (submitted). The limit of detection was about 10 pmol/g globin (40 mg globin samples) and the coefficient of variation for duplicate preparation (on different occasions) and measurement was between 5 and 16%.

2.5. Flow cytometric MN assay

The procedure for sampling, fixation, staining and flow cytometric analysis of erythrocytes from mouse peripheral blood and rat bone marrow, was the same as

earlier reported [8,15,22]. Essentially, after anesthetization about 100–200 μl of mouse peripheral blood was drawn from orbital veins into heparinized tube using a Pasteur pipette. Triplicate samples of 6 μl blood were layered on 1 ml of 65% Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) solution in phosphate buffered saline (PBS) and centrifuged for 20 min at 600 × g. Rats were anesthetized and sacrificed and, by a syringe, bone marrow from both femurs was flushed into a conical tube (4.5 ml; 12/75 mm, Greiner, Kebo, Spånga, Sweden) with 2 ml of RPMI-medium (Gibco, UK), centrifuged and resuspended in 120 μl PBS. Triplicate samples of 30 μl rat bone marrow suspension were layered on 1 ml of 65% Percoll solution in PBS, as above, and centrifuged. Supernatants from mouse as well as rat samples were carefully aspirated, leaving a pellet of erythrocytes and some nucleated cells. The pellets obtained were suspended in 30 μl Sörensen's phosphate buffer, 0.05 M, pH 6.8, with 1.5 mg/ml sodium dodecyl sulfate (SDS; Sigma, St. Louis, MO, USA) included. After overnight storage at 4 °C, the fixative was removed and staining buffer was added under gentle vortexing. The staining buffer was prepared by adding 500 μl Hoechst 33342 stock (HO342; Molecular Probes, Pitchford, OR, USA; 500 mM in distilled water) and 50 μl Thiazole Orange stock (TO; Molecular Probes, Eugene, USA; 1 mg/ml in methanol) to 100 ml PBS. The samples were stained with 1 ml staining buffer during 1 h, in the darkness, mixed every 15 min. Afterwards, the flow cytometric analysis and calculation of PCE and micronucleated PCE (MPCE) frequencies were performed as described by Grawé et al. [22]. The coefficient of variation for the MN assay (pooled samples) was 14%.

2.6. Statistical methods

Relations between adduct levels and administered doses as well as between MN frequencies and in vivo doses were assessed by linear or linear-quadratic regression, using SAS® software [23]. Calculated parameters are presented together with either 95% confidence interval (CI), standard deviation (S.D.) or P-value. The responses of the MN frequencies as a function of the in vivo dose of GA obtained by i.p. injection of synthetic GA or as a metabolite of AA (data from an earlier study [8]), were calculated on the basis of the average adduct level of all animals in each dose group. The hypothesis of the same linear-quadratic dose–response curve independent of mode of exposure was tested by a likelihood ratio test.

3. Results

3.1. Synthesis of GA

Previously, GA has been prepared by reaction of AA with alkaline hydrogen peroxide under strictly controlled conditions of pH [24,25]. Our way of synthesizing GA, via direct epoxidation of AA with DMD avoiding the involvement of peroxides, has the advantage that the labile GA can be isolated simply by evaporation of the reactive material and solvent. The GA was obtained essentially pure (95%) and its ^{1}H NMR was fully consistent with the structure and with previously reported data [24,25].

3.2. Hb adduct levels

Analysis of GA adducts in Hb in blood samples from GA-treated rats and mice showed linearly dose-dependent adduct level increments that were significantly different in the two species ($P = 0.003$) (Fig. 3). The increase in adduct level per unit dose of GA (in mmol/kg body weight) were 82 nmol/g globin (95% CI: 76–90) for rats and 61 nmol/g globin (95% CI: 55–67) for mice. Thus, the adduct levels in rats were 32% (95% CI: 12–65) higher per administered dose of GA than the levels in mice. Average adduct level in control animals was for mice 0.036 nmol/g globin (S.D. = 0.004) ($n = 4$) and for rats 0.012 nmol/g globin (S.D. = 0.003) ($n = 4$).

3.3. The MN test

The result from the MN test of peripheral blood from mice showed a linear-quadratic increase in the frequency of MPCE with administered dose of GA. Combined results from the pilot study and the main study are shown in Fig. 4.

The results from the MN test of rat bone marrow showed a non-monotone dose–response. At the administered doses 0.7 and 0.9 mmol/kg body weight there was a weak, but significant ($P = 0.001$ for combined

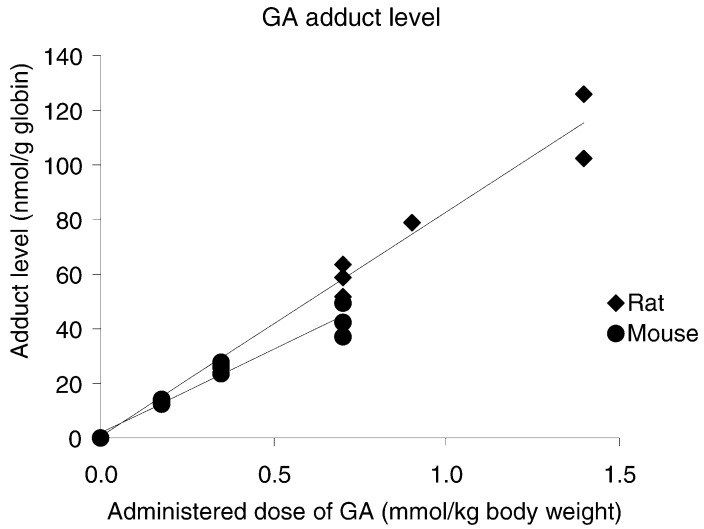

Fig. 3. GA adduct levels in rat and mouse after GA treatment.

results from the pilot and main studies, $P = 0.02$ for results from the main study), increase in the frequency of MPCE while at the highest given dose, 1.4 mmol/kg body weight, the MPCE frequencies were approximately similar to the background values. In Fig. 5 results from the main study and the pilot study are shown.

In both mice and rats the frequency of PCE was monitored as a measure of bone marrow toxicity impairing cell proliferation. In both species the treated animals showed, in comparison to the control animals, a decrease in the PCE frequency. In the mice the decrease at the administered dose 0.7 mmol/kg body weight was about 40%, the same as in the pilot study

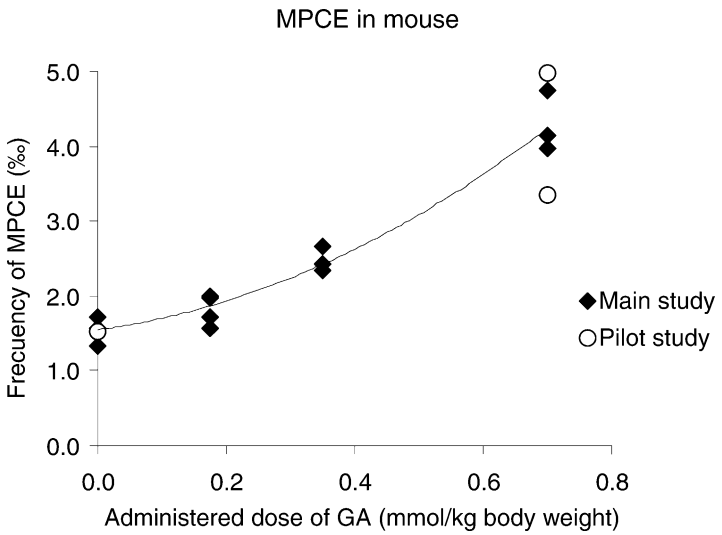

Fig. 4. MN frequencies, measured as frequencies of MPCE, in mouse after GA treatment. Combined results from the pilot study and the main study.

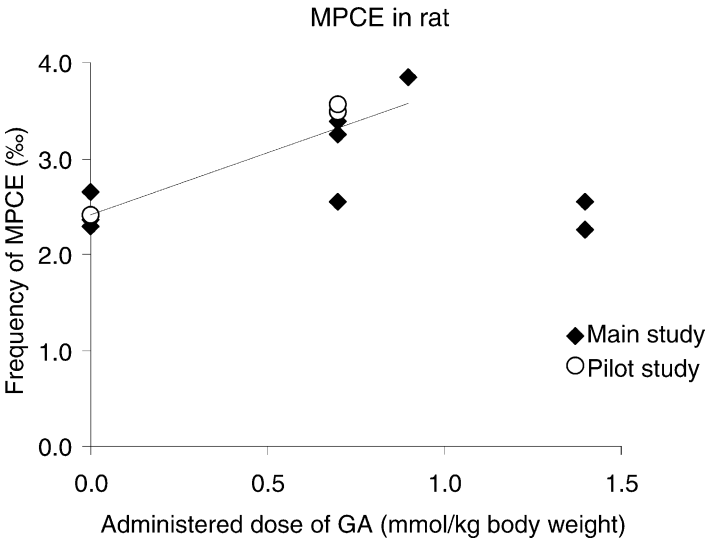

Fig. 5. MN frequencies, measured as frequencies of MPCE, in rat after GA treatment. Combined results from the pilot study and the main study.

(Fig. 6). In the rats, however, the decrease was more pronounced in the main study than in the pilot study. For rats given 0.7 mmol/kg body weight the decrease in PCE frequency was about 40%, compared with 20% in the pilot study. At higher administered doses, the PCE frequencies in rats were as low as one third of the background values (Fig. 7). It is known from our earlier work that while MPCE frequencies are highly reproducible between experiments, PCE frequencies are more variable. The results show that in mice the increase in MN is apparent even at doses producing no or little bone marrow toxicity. In the rat, however, the modest increase in MN is observed at doses with pronounced bone marrow toxicity. This indicates that

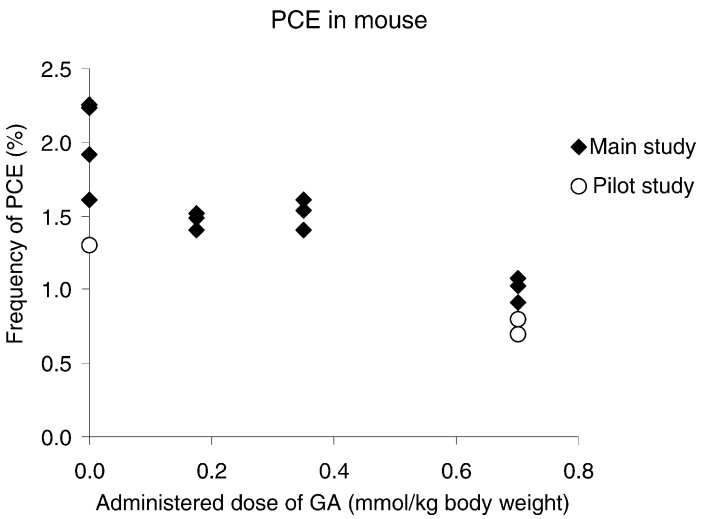

Fig. 6. Frequencies of PCE, an indication of toxic effect, in mouse after GA treatment. Results from both the pilot study and the main study.

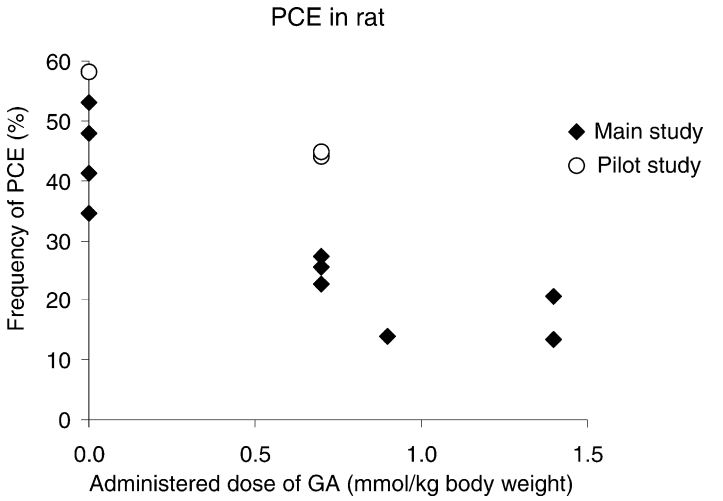

Fig. 7. Frequencies of PCE, an indication of toxic effect, in rat after GA treatment. Results from both the pilot study and the main study.

the measured MN frequencies in rats may be underestimated due to reduced proliferation in the bone marrow, particularly at the highest administered doses of GA.

3.4. Body weight

The animals were weighed before and after the treatment in the main study and the result showed that the rats were, in contrast to the mice, loosing body weight during the treatment (Table 1). The treated rats lost between 2 and 8% of their body weight 24 h after treatment. This is seen as a symptom of systemic effects caused by the treatment of GA.

Table 1
Average difference in body weight for each dose group of mice and rats after GA treatment

Administered dose (mmol/kg)	*n*	Weight difference—mice		Weight difference—rats	
		g	%	g	%
Control	4	0.2	0.8	0.5	0.2
0.18	4	0.0	0.0	–	–
0.35	3	0.4	1.3	–	–
0.7	3	0.1	0.3	−7.7	−2
0.9	1	–	–	−14	−5
1.4	2	–	–	−26	−8

4. Discussion

In both mice and rats GA induced linearly dose-dependent increases in Hb-adduct levels (Fig. 3). The GA adduct levels (reflecting in vivo doses) in rats were significantly (32%) higher per administered dose than in mice. This implies that the detoxification of GA is faster in the mouse than in the rat. This conclusion is based on the assumption that the reactivity of GA toward N-terminal valine is approximately the same in rats and mice (shown, e.g. for ethylene oxide by Granath et al. [9].

The flow cytometric method used for the determination of MN frequencies is very stable over time, as shown both in studies involving several repeat samplings over a period of more than 3 months [26], and when comparing identical exposures and animal strains in experiments made several years apart [27,28]. Thus, experiments done several months apart may be quantitatively compared. Here we have utilized this reproducibility over time to combine the results of pilot and main experiments with GA, and in order to compare two ways of establishing GA doses in vivo (AA treatment and GA treatment). The combination of the GA data in the present study is of advantage especially in the study of rats, where a small number of animals were used.

The incremental MN frequencies per administered dose of GA in mice were compatible with

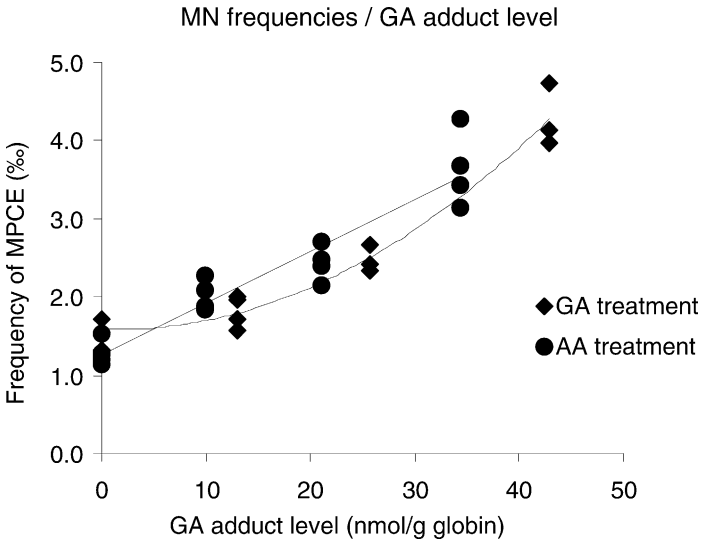

Fig. 8. MN frequency per unit of in vivo GA dose in mouse after AA treatment (the previous study) as well as after GA treatment (the present study). On the *x*-axis are the measured GA adduct levels (mean values from the different dose-groups) and on the *y*-axis the frequency of MPCE (individual values).

linear-quadratic dose-dependence (Fig. 4). In the rats, no positive dose–response relationship was obtained, there was a weak increase in MN frequency at the two lowest but no increase at the highest administered GA dose (Fig. 5). Adduct levels (in vivo doses) of GA were, however, higher in rats than in mice over the whole range of administered doses.

At the highest administered doses the rats showed symptoms of systemic toxicity, decreased erythrocyte proliferation in the bone marrow together with loss of body weight. The low MN frequencies at these administered doses of GA in rats are probably due to these toxic effects. The genotoxic effect of GA in rat, measured by MN frequencies in the bone marrow PCE, may have been overshadowed by the systemic effects caused by GA. The MN assay, therefore, does not seem to be suitable for the measurement of genotoxic effects caused by GA in the rat. For performing a comparison of genotoxic potency of GA in mice and rats another assay for measuring genotoxic endpoints should be employed.

In the preceding study of mice and rats treated with AA, Hb adducts from the parent compound AA and the metabolite GA were measured together with MN frequencies [8]. The AA treatment of mice in that study led to in vivo GA doses (measured as GA adduct levels) similar to those in the present study. A comparison between the present and the preceding studies with regard to MN frequency per unit of in vivo dose of GA in mice, shows a similar response to the in vivo GA dose whether it results from metabolism of AA or from administered synthetic GA (Fig. 8). The shapes of the two dose response curves are indicated to be different, however. The curve corresponding to the MN frequency per in vivo dose of GA after AA treatment is linear while the curve corresponding to the MN frequency per in vivo dose of GA after GA treatment shows a linear-quadratic shape. The latter was also the case for the curve corresponding to MN increase per administered GA dose (Fig. 4).

The regression coefficients when fitting a simple linear or a linear-quadratic dose response curve to the observed MN frequencies as a function of GA adduct levels in mice are shown in Table 2. As can be seen the linear model yields almost identical slopes. However, when fitting a linear-quadratic model, the same linear-quadratic model does not apply to the two modes of exposure ($P = 0.02$). In fact, the response after AA treatment is almost purely linear, while the response after GA treatment is almost purely quadratic. This may reflect a true difference in the response due to the way of exposure but one should not,

Table 2
Regression coefficients when fitting the same simple linear model or the same linear-quadratic model to the observed MN frequencies as a function of measured GA adduct level in mice, as shown in Fig. 8

Parameters	AA treatment		GA treatment	
	Regression coefficient	*P*	Regression coefficient	*P*
Linear model				
Background (intercept)	1.26		1.27	
Linear (slope)	0.066	<0.0001	0.062	<0.0001
Linear-quadratic model[a]				
Background (intercept)	1.32		1.59	
Linear term	0.049	0.004	−0.005	0.69
Quadratic term	0.0005	0.31	0.0016	<0.0001

[a] The hypothesis of identical linear-quadratic dose–response independent of mode of exposure, can be rejected ($P = 0.02$).

at this stage, absolutely exclude unknown systematic errors.

It should be noted that the agreement between the two studies with different treatments includes that, in the previous study, GA is formed through metabolism of AA and that in the present study, the in vivo GA dose is received through i.p. injection of synthetic GA. This fact will have an impact on the dose rate, i.e. the concentration, of GA in vivo, in the two studies. A higher dose rate (as obtained by i.p. injection of the genotoxic synthetic GA) might, for instance, influence the rate of DNA repair. Further studies would be needed for a clarification of the discrepancy between the two studies.

To conclude, the results from the present study in combination with the preceding study show that the increase in MN in AA-treated mice could be ascribed to the formation of the genotoxic metabolite GA. It seems that the in vivo doses of AA in the previous study do not contribute to the observed genotoxic effects from AA treatment. The results support the hypothesis that it is the metabolite GA that is the predominant genotoxic agent following exposure to AA.

A similar comparison for rats could not be made since, in the previous study AA-treated rats exhibited no MN increase (most probably due to too low in vivo GA doses); in the present study we could not estimate any dose response curve for MN increase per in vivo dose of GA (due to the probably underestimated MN frequencies in the rats given the highest administered dose of GA).

In a recent paper Park et al. [29] presented strong indications of glutathione depletion playing an essential role in the induction of cell transformation by AA. The authors collect a number of arguments against a causative action of reaction with DNA of the metabolite GA in the genotoxic effects of AA. In this context measurements of doses as in the present study, are most useful for the elucidation of genotoxic mechanisms.

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