RESEARCH ARTICLE

Biological effects of acrylamide after daily ingestion of various foods in comparison to water: A study in rats

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Scope: Acrylamide (AA), classified as a genotoxic carcinogen, is generated by heating foods. We studied whether the food matrix modulates bioavailability and/or biotransformation and investigated kinetics and biological effectiveness of AA in rats.
Methods and results: AA was given to the animals at a daily intake level of AA containing foods for up to 9 days, resulting in an exposure of 50 or 100 μg AA/kg body weight (b.w.)/day. Positive controls received the same dosages of AA in water, negative controls just water. As biomarkers urinary mercapturic acids, hemoglobin adducts, plasma levels of AA and glycidamide (GA) and DNA integrity in white blood cells and hepatocytes were measured. Altogether, no significant differences in bioavailability of AA from water and the different food matrices were observed. Only with bread crust, biomarkers indicated a slightly reduced bioavailability. Monitoring glycidamide valine adduct adducts did not provide evidence for treatment-related significantly enhanced GA-haemoglobin adduct formation in blood although glycidamide mercapturic acid excretion in urine indicated significant GA formation.
Conclusions: The results suggest AA at dietary intake levels, exceeding estimated human mean intake by a factor of at least 100 to become detoxified in Sprague–Dawley rats to a major extent through glutathione coupling.

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1 Introduction

Acrylamide (AA) can be generated in foods upon heating from reducing carbohydrates and asparagine by Maillard reaction chemistry. After dietary uptake, it is conceived to undergo in part metabolic transformation into the proximate genotoxic metabolite glycidamide (GA). AA has been classified as class 2A carcinogen (probably carcinogenic to humans) by IARC [1].

Margins of exposure (MOEs) for AA have been calculated, based on the doses correlated to a low, typically 10% response for tumour formation (bench mark dose at the lower 95% confidence limit, BMDL10) in male and female rats. These are put in relation to average or high dietary exposure data from numerous national estimates, reflecting the low and high ends of the dietary exposure ranges reported. The resulting MOEs range between less than 50 to up to about 1000, depending on the critical tumour lesion taken as point of departure and on average *versus* high-exposure estimates [2]. MoEs of 10 000 and above are viewed of low concern from a public health point of view, but concern increases, the lower the MoE [3]. This would especially apply to an MoE of less than 1000, as has been allocated to AA, as well as to some other genotoxic carcinogens.

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Abbreviations: AA, acrylamide; **AA-DW,** AA uptake *via* drinking water; **AAMA,** acrylamide mercapturic acid; **AAVal,** acrylamide valine adduct; **BC,** bread crust; **b.w.,** bodyweight; **FFR,** French fries reconstituted; **FFS,** French fries sliced; **FPG,** formamidopyrimidine DNA glycosylase; **GA,** glycidamide; **GAMA,** glycidamide mercapturic acid; **GAVal,** glycidamide valine adduct; **GB,** gingerbread; **Hb,** haemoglobin; **MA,** mercapturic acid; **MOE,** margins of exposure; **PFPTH,** pentafluorophenylthiohydantoin; **SD rat,** Sprague–Dawley rat; **TI,** tail intensity

In foodstuffs, AA is formed from asparagine during the Maillard reaction *via* Strecker degradation in the presence of reducing sugars. Several pathways have been proposed for the formation of the proximal intermediate 3-aminopropionamide, generating AA by heat-induced elimination of ammonia. Among the foods found to contain substantial amounts of AA are potato products, such as fried potatoes or French fries (median content 240 μg AA/kg (range: <10–3260), gingerbread (GB) (300 μg AA/kg; range: <10–7834) and bread (15 μg AA/kg; range: <30–70) where the main part of AA is located in the crust [4].

Studies in rats indicate that AA orally administered *via* drinking water is rapidly and extensively absorbed from the gastrointestinal tract. AA and AA-related metabolites are widely distributed among tissues with no evidence for an accumulation [5–8].

AA is presumed to exert its genotoxic activity *via* metabolic transformation into the genotoxic metabolite GA, primarily by cytochrome P450 2E1 [9–12]. GA is known to be mutagenic in bacteria [13] and mammalian cells [14–20]. On the contrary, AA without metabolic activation has not been found to be mutagenic or genotoxic at biologically relevant concentrations [13]. AA doses in the range of 36–54 mg/kg body weight (b.w.) given to Sprague–Dawley (SD) rats induced significant DNA damage in leukocytes, brain and testes as monitored by the comet assay [21]. At still lower doses (1 mg AA/kg b.w.), induction of micronuclei was reported [22]. In the earlier rat studies [10], *N*7-GA-guanine adducts were detected at doses of 46 mg AA/kg b.w. by intraperitoneal injection. After treatment of adult mice with 50 mg AA/kg b.w., formation of *N*7-GA-guanine and, to a minor extent, also of *N*3-GA-adenine adducts was reported for liver, lung and kidney [23]. Formation of *N*7-GA-guanine adducts close to the LOD (one adduct/10^8 nucleotides) has been reported in liver cells from Fischer 344 rats, given single oral doses of 100 μg AA/kg b.w. in drinking water by gavage or in the diet [6].

Another mode of interaction with biomolecules concerns binding of AA and GA to nucleophilic regions in proteins and peptides, such as amino and sulfhydryl groups. Hemoglobin (Hb) adducts are often used as validated biomarkers to monitor internal exposure to electrophilic xenobiotics [24]. Hb adducts of AA with the *N*-terminal valine (Val) serve as biomarkers for cumulative exposure over the average life span of the erythrocytes [25–28]. A recent study showed correlations between glycidamide valine adduct (GAVal)/acrylamide valine adduct (AAVal) ratios and phases I and II enzyme genotypes [29]. AA and GA are detoxified in the mammalian organism by conjugation to glutathione. Glutathione adducts are converted to the respective mercapturic acid (MA) metabolites *N*-acetyl-*S*-(2-carbamoylethyl)cysteine (AAMA, acrylamide mercapturic acid), *N*-acetyl-*S*-(1-carbamoyl-2-hydroxyethyl)cysteine and *N*-acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)cysteine (GAMA, glycidamide mercapturic acid) to be excreted in the urine [30–32].

The MAs AAMA and GAMA have been used as biomarkers of internal AA exposure, GAMA serving as an indicator for biotransformation of AA to GA. A urinary GAMA/AAMA ratio of 0.1 has been observed for humans [30], whereas for rats and mice, ratios of 0.2 and 0.5, respectively, were reported, indicating that rodents more efficiently biotransform AA to GA than humans [31, 32].

Only few data exist at present on bioavailability of AA from food. A study in rats reported 32–44% bioavailability from food spiked with AA [6]. However, since AA was added in this study to the diet, it might have been scavenged in part already by Michael reaction with nucleophilic food constituents and the data might thus not be representative for intrinsic AA contents in food. On the contrary, approximately complete bioavailability of AA from crisp bread in mice was evidenced in another study, showing that the urinary excretion of AA and related metabolites after crisp bread feeding was identical to subcutaneous injection [33]. After consumption of potato crisps containing a high level of AA by volunteers, equivalent to 12.4 μg AA/kg b.w., urinary AA and metabolite levels were monitored [30]. Total AAMA and GAMA excretion of probands who consumed potato crisps was comparable to that of a volunteer who received a single dose of deuterium-labelled AA in drinking water [32].

A prime question addressed in this study was to determine whether specific food matrices affect bioavailability and biological activity of AA in rats. A range of matrices was chosen, represented by French fries, sliced from potatoes with normal plant texture (FFS, French fries sliced) or reconstituted from potato starch representing a dough-like texture (FFR, French fries reconstituted), by bread crust (BC) and by GB. Animals received identical amounts of AA in the selected diets or in water for 1–9 days at a dosage of 50 or 100 μg AA/kg b.w. As biomarkers, the MAs of AA (AAMA) and GA (GAMA) excreted within 24 h subsequent to the last feeding were measured (LC/MS-MS). Since in rats within 24 h more than 90% of MAs are excreted in the urine [34], monitoring of MAs should allow to focus on short-term exposure because accumulation is not expected to occur. In addition, Hb adducts of AA and its genotoxic metabolite GA (GC/MS) were determined as biomarkers indicative not only for bioavailability under continuous exposure, but also for bioactivation of AA to genotoxic GA. The results were expected to yield another major outcome of this study, giving a measure of cumulative overall internal exposure of AA and GA during the experimental period chosen in different compartments of the organism. Moreover, by exploiting these short- and long-term biomarkers, we expected to gain information on food borne-modulating effects, including potential adaptive responses of the organism. In addition to the biomarkers chosen to monitor short-term and cumulative exposure, we also monitored DNA damage in peripheral leukocytes and in hepatocytes, using alkaline single-cell gel electrophoresis (comet assay)

with post-treatment by formamidopyrimidine DNA glycosylase (FPG). This enzyme is a constituent of an important bacterial base excision repair system. In addition to recognizing abasic sites and removing oxidatively damaged DNA bases, it also removes *N*7-guanine alkylation, the main DNA lesion expected to occur when GA reacts with DNA.

In addition, short-term toxicokinetics were studied in a satellite experiment encompassing dietary AA uptake *via* French fries in comparison to uptake by gavage in drinking water. AA and GA levels in blood were determined at specified intervals for up to 4 h after AA dosing.

2 Materials and methods

2.1 Chemicals

Chemicals used were of analytical grade. AA was obtained from Merck (Darmstadt, Germany). D_5-AA (CDN Isotopes, Canada) and $^{13}C_3$-GA (Toronto Research Chemicals, Canada) were used for internal standardization. FPG was provided by A. R. Collins (University of Oslo, Norway). Agarose (low and normal melting) was from Serva Electrophoresis GmbH (Heidelberg, Germany). Pentafluorophenylisothiocyanate was from Fluka (Buchs, Switzerland). AAValPFPTH (PFPTH, pentafluorophenylthiohydantoin), D_7-AAValPFPTH, GAValP FPTH and D_7-GAValPFPTH were synthesized according to Bergmark *et al.* and Paulsson *et al.*, with minor modifications [26, 35].

The MAs of acrylamide (AAMA) and glycidamide (GAMA) as well as the D_3-labelled analogues were synthesized as described in the literature [36]. Briefly, AA, AA-2,3,3-D_3 (Cambridge Isotope Laboratories, USA), respectively, GA and GA-2,3,3-D_3 were reacted with *N*-acetyl-*S*-cysteine in the presence of sodium ethylate as a basic catalyst. GA and GA-2,3,3-D_3 were synthesized by H_2O_2 oxidation of acrylonitrile, respectively, acrylonitrile-2,3,3-D_3, following a method devised by Payne and Williams [37]. The products were characterized by MS (turbo ion spray; direct infusion and negative ionization mode) and ^{1}H NMR. Purity was ascertained to be $>95\%$ for all substances.

Mass spectrum of AAMA: *m/z*, 233.0 (M−H)-, 161.7, 104.0 and 58.1

D_3-AAMA: *m/z*, 236.0 (M−H)-, 161.7, 106.9 and 59.0

GAMA: *m/z*, 249.0 (M−H)-, 120.0, 102.0 and 74.8

D_3-GAMA: *m/z*, 252.0 (M−H)-, 122.9 and 105.0

^{1}H NMR (400 MHz, 273 K, (ppm)):

AAMA:δ = 1.75–1.87 (s, 3H, CH_3), 2.16–2.35 (m, 2H, S-CH_2), 2.52–2.86 (m, CH_2-S), 6.67–6.82 (s, H, NH2), 7.39–7.46 (s, H, NH_2), 7.33–7.39 (d, 1H, NH)

D_3-AAMA: 1.76–1.87 (s, 3H, CH_3), 6.67–6.83 (s, H, NH_2), 7.39–7.51 (s, H, NH_2), 7.30–7.39 (d, 1H, NH), 2.70–2.79 (d, 1H, cys β), 2.87–2.97 (d, 1H, cys β′), 2.16–2.28 (m, 1H, CD_2-CDH)

GAMA: 1.76–1.91 (s, 3H, CH_3), 7.05–7.25 (s, H, NH_2), 7.44–7.56 (s, H, NH_2), 7.26–7.44 (d, 1H, NH), 6.14–6.40 (s, 1H, OH)

D_3-GAMA: 1.84–1.87 (s, 3H, CH_3), 7.41–7.62 (d, 1H, NH), 6.20–6.30 (s, 1H, OH)

2.2 Food preparation

French fries (3 kg/batch) were prepared by frying potato sticks from raw potatoes (FFS) or were reconstituted by frying potato starch dough (FFR) by Deutsches Institut für Lebensmittelforschung, Quakenbrück, Germany. BC was obtained as a 5 mm crust layer from wholemeal rye loaf bread, prepared following a modified recipe and procedure (Institut für Lebensmitteluntersuchung, Nuthetal, Germany). To enhance AA formation during the baking process, ammonium bicarbonate (1% w/w) and invert sugar cream (4% w/w relating to the meal proportion) were added. GB (provided by Institut für Lebensmitteluntersuchung), was prepared following a standard recipe utilizing a wheat and rye flour mixture (21.7 and 9.3% w/w), invert sugar cream (25.2% w/w) and glycose syrup (2.8% w/w). After preparation, foods were chopped into small pieces and homogenously mixed. Aliquots were shrink wrapped and stored at −20°C. AA content in French fries was 2850 μg/kg (FFS and FFR), in BC 1400 μg/kg. AA contents of FFS, FFR and BC were determined by collaborating partner labs (DIL Quakenbrück and ILU Nuthetal, Germany) and in parallel by a commercial lab (Eurofins, Hamburg, Germany). Values did not deviate by more than 10%. GB was cooled by liquid nitrogen before being homogenized and sent to several laboratories for AA analysis in dry ice. AA contents found in GB were of considerably higher variability than in the former foods. A mean AA content ($n = 15$) of 4150 ± 800 μg AA/kg was assessed.

2.3 Animal treatment

2.3.1 Feeding study

All animals were maintained under standard conditions in accordance with the animal protection standards. Male SD rats (287.3 ± 15.5 g) were adapted to housing conditions (20–22°C) over 10 days and were trained to ingest defined amounts of food in a metabolism cage (Tecniplast, Buguggiate, Italy) within 3 h after overnight fasting (between 8 and 11 a.m.). Reproducible AA uptake from food was achieved by supplying aliquots of approximately 4–11 g FFS, FFR, GB and BC to make up for the target dosages, 50 μg AA/kg b.w./day for BC and 100 μg AA/kg b.w./day for FFS, FFR and GB. Three hours after the beginning of the treatment, three rats of each group were put into standard macrolon cages and received standard diet *ad lib* (ssniff Spezialdiäten GmbH, Soest, Germany) with an AA content <30 μg/kg (AA determination by Eurofins) for

another 7 h. Thereafter, they were set again on overnight fasting. To adjust the AA uptake *via* drinking water (AA-DW group) to the time interval required for uptake of AA over food, doses of 50 and 100 μg AA/kg b.w. were administered in two portions of approximately 0.5 mL each (dosing solutions 17 μg AA/mL and 25 μg AA/mL, respectively) by gavage under moderate isoflurane anesthesia applied for ~60 s for tranquilization to lower the risk of injuries. Animals in all other treatment groups and the untreated controls were isoflurane treated in the same way to avoid any confounding effect of anesthesia. Animals had free access to drinking water. The described dosing procedure was repeated according to the required number of treatment days. At the respective last treatment day, animals were kept in metabolism cages after AA food uptake and urine and feces were collected during 24 h. After 24 h, animals were sacrificed by cervical dislocation and blood and liver were isolated.

2.4 Satellite experiments

2.4.1 Influence of anaesthesia

The potential influence of moderate isoflurane anesthesia on the AA metabolism was investigated by applying two doses of 100 μg AA/kg b.w. by gavage on two consecutive days with or without anesthesia (two animals each).

2.4.2 Single high-dose experiments

In a second satellite experiment, three animals *per* group received the total AA in a single shot of 0.45, 0.9 or 10 mg AA/kg b.w. by gavage, respectively (approximately 0.5 mL of dosing solutions containing 0.25, 0.5 and 5 mg AA/mL). The animals were held and examined as described before.

2.4.3 Short-term toxicokinetics

Short-term uptake kinetics of AA was measured in four rats, given a single dose of 100 μg AA/kg b.w. (dosing solution 50 μg AA/mL) *via* gavage. In a parallel experiment, a second group of four rats was fed FFS corresponding to 100 μg AA/kg b.w. within an uptake time of 15 min. For the determination of AA and GA in serum, 12 blood samples in total were taken from each treatment group by retrobulbar puncture under isoflurane anaesthesia, covering four time points (30, 60, 120 and 240 min after AA uptake), resulting in three blood samples *per* time point.

2.4.4 AA- and GAVal-adduct determination

N-terminal AAVal and GAVal Hb adducts were determined by GC/MS with the mass spectrometer in negative chemical ionization mode [27, 28, 35]. An Agilent Technologies (Santa Clara, CA, USA) 6890N Network GC System was used (column: DB-XLB, 30 m × 0.25 mm id, 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA); helium flow, 1.3 mL/min; injector, PTV injector used in pulsed splitless mode at 250°C; injection volume, 1 μL; temperature program: 90°C for 1 min, 25°C/min to 210°C, 10°C/min to 240°C and 25°C/min to 310°C for 5 min), coupled to an 5973 inert (Agilent Technologies) mass selective detector device (reactant gas, methane (45%); ion source temperature, 150°C; quadrupole temperature, 150°C and transfer line temperature, 280°C). Hb adducts were determined as PFPTH derivatives of the *N*-terminal valine adducts, obtained by a modified Edman degradation procedure as described elsewhere [27, 28, 35].

As internal standards for AAVal and GAVal, deuterium-labelled D_7-AAValPFPTH and D_7-GAValPFPTH were used. These standards were synthesized following described procedures, with minor modifications [26, 38]. GAValPFPTH and D_7-GAValPFPTH were subjected to acetonide formation to improve gas chromatographic properties as described by Paulsson *et al.* [35]. The method resulted in LOQs of 0.9 and 2.3 pmol/g Hb for AAVal and GAVal, respectively. The LODs were 0.3 pmol/g Hb for AAVal and 0.6 pmol/g Hb for GAVal. Precision did not exceed a 9.6% deviation for AAVal and 12.8% for GAVal, accuracy ranged from 2 to 8.7% deviation for AAVal and from 7.2 to 14.6% for GAVal, respectively. *R*-values were 0.999 for both AAValPFPTH and GAValPFPTH. These characteristics are in the same range as those reported earlier by other groups [28, 39–41].

2.4.5 AAMA and GAMA determination in urine

AAMA and GAMA were determined according to a method devised by Boettcher *et al.* [42] with slight modifications. Urine samples were thawed and vortexed. Aliquots of 1–2 mL were diluted with ammonium formate buffer (50 mM, pH 2.5) (total volume of 8 mL) and internal standards (D_3-AAMA, D_3-GAMA) were added. The pH was adjusted to 2.5 (4 N HCl) and samples were centrifuged at 3000 × *g* for 15 min. The supernatant was applied to an SPE column (Isolute ENV$^+$, 10 mL; 100 mg; Separtis, Grenzach-Wyhlen, Germany), preconditioned with 4 mL methanol, 2 mL deionized H_2O and 2 mL HCl (pH 2.5). The column was washed consecutively with 2 mL HCl (pH 2.5) and 1 mL HCl (pH 2.5) containing 10% methanol v/v and was dried by water jet pump. Analytes were eluted by 1.85 mL methanol containing 1% formic acid. The eluate was evaporated to dryness under nitrogen and the residue was dissolved in 1 mL formic acid (0.1% v/v).

LC was performed using a Perkin Elmer Series 200 HPLC system coupled to a SCIEX API 2000 triple quadrupole mass spectrometer equipped with a turbo ion spray source using multiple reaction monitoring negative

ionization mode and a 10-port valve. Sample volumes of 50 μL were injected onto a reversed phase HPLC column (Phenomenex Luna C8 (2), 150 mm × 4.6 mm, 3 μm particle size) equipped with a guard column. The isocratic mobile phase consisted of formic acid (0.1%) with 5% ACN at a flow rate of 300 μL/min. The eluate cut from 6 to 13 min was directed into the electrospray interface. The MAs were quantified by isotope dilution with reference to the afore-described stable isotope-labelled standards. The LODs and LOQs were 4 and 8 pmol, respectively, for AAMA and 8 and 12 pmol, respectively, for GAMA. *R*-values were 0.998 for both AAMA and GAMA. The sensitivity of the method is in line and compares well with respect to these characteristics with published methodology [42, 43].

2.4.6 AA and GA determination in urine, blood and feces

Urine samples were thawed and sample aliquots (1–3 mL), spiked with the internal standards (D_5-AA; $^{13}C_3$-GA). After vortexing and centrifugation (3000 × *g* for 15 min), the supernatant was applied to SPE columns (Isolute ENV$^+$, 500 mg; 6 mL), preconditioned with 4 mL methanol and 2 × 4 mL H_2O. The column was washed with 4 mL H_2O and analytes eluted with 2 mL of 60% methanol in H_2O v/v. The eluate was concentrated to approximately 1 mL in a nitrogen stream. Briefly, 50 μL of this solution was injected into the LC/MS-MS.

Purification and preparation of the serum samples were performed according to Twaddle *et al.* [44] with slight modifications. Briefly, frozen serum samples were thawed and vortexed. Aliquots of 100–200 μL were spiked with the internal standards (D_5-AA; $^{13}C_3$-GA) and diluted to a total volume of 400 μL. After vortexing, samples were applied to an SPE column (Isolute ENV$^+$, 10 mL; 100 mg), preconditioned with 4 mL methanol and 4 mL H_2O. The column was washed with 0.4 mL H_2O and 0.4 mL H_2O containing 2% v/v methanol. The analytes were eluted with 1 mL H_2O containing 10% ACN. The eluate was evaporated to 100 μL under nitrogen stream. Aliquots (90 μL) were injected into the LC/MS-MS. To determine the LOD of GA in rat serum, serum samples (200 μL) of untreated rats were spiked with 0.54–0.72 ng $^{13}C_3$-GA, corresponding to the concentrations of 30–40 nM. A threefold background noise signal was found with serum samples containing 35 nM $^{13}C_3$-GA and this was defined as LOD.

Fecal samples (24 h collection, weight recorded) of each animal were thawed and homogenized by mortar and pestle. After transfer to a 50 mL polyethylene vial, internal standards and H_2O (20–40 mL) were added. Suspensions were vortexed for 5 min and aliquots of the supernatant (10 mL) obtained by centrifugation (3000 × *g*; 60 min; 4°C) applied to SPE columns (Isolute ENV$^+$, 500 mg; 6 mL), preconditioned with 4 mL methanol and 2 × 4 mL deionized H_2O. After washing with deionized water (4 mL), analytes were eluted with 2.5 mL of 60% methanol in water v/v and the eluate concentrated to 1 mL by nitrogen stream. Aliquots (90 μL) of this solution were injected into the LC/MS-MS system.

A SCIEX API 3200 MS/MS system was used with an ESI source in multiple reaction monitoring mode for the analysis of positive ions. ESI needle voltage, 5500 V. Nitrogen as nebulizer and turbo heater gas (200°C), pressure, 15 or 30 psi, respectively. The curtain gas and the collision gas were set to 10 psi and five arbitrary units, respectively. Compound-specific MS parameters were optimized by Quantitative Optimization Wizard (SCIEX Analyst 1.4.2TM software) using continuous injection (syringe pump) into the mass spectrometer. The analytes were quantified by isotope dilution using the corresponding stable isotope-labelled substances. Transitions: AA: 72 → 55; D_5-AA: 75 → 58; GA: 88 → 44; $^{13}C_3$-GA: 91 → 47 with correlation coefficients (R^2) of 0.985 for AA and GA. The LODs and LOQs were 0.14 and 0.7 pmol for AA and 12 and 23 pmol for GA (absolute amounts).

Analytes were HPLC separated using a Jasco HPLC-system (PU-2080 plus HPLC-pump; DG-2080-53 degasser; LG-2080-02 ternary gradient unit; AS-2055 plus auto-sampler; Jasco GmbH, Groβ-Umstadt, Germany) equipped with a Merck LiChrospher RP18 endcapped column (250 × 4 m; 5 μm particle size), mobile phase 0.1% formic acid v/v and 1% methanol v/v (isocratic, flow rate: 0.5 mL/min). The eluate cut from 5 to 15 min was directed into the ESI source.

For the determination of AA and GA in serum and urine, the previously described LC/MS-MS system was used with modifications. Nitrogen as nebulizer and turbo heater gas (450°C) was applied at a pressure of 20 and 40 psi, respectively. Transitions: AA: 72 → 55; D_5-AA: 75 → 58; GA: 88 → 71; $^{13}C_3$-GA: 91 → 74. The LODs and LOQs were 0.6 and 1.2 pmol, respectively, for AA and 3 and 8 pmol, respectively, for GA (absolute amounts).

For these samples, a Phenomenex Luna C8 (2) (150 mm × 4.6 mm, 3 μm particle size) column was used, equipped with a guard column, mobile phase: methanol containing 0.05% formic acid (flow rate, 0.3 mL/min). The analytes were eluted with a gradient from 1 to 20% methanol over 20 min. The eluate cut from 6 to 17 min was directed into the ESI source.

2.4.7 DNA damage in liver and white blood cells

Liver cells were isolated according to Kamp *et al.* [45]. Briefly, a piece of the left liver lobe was minced with a pair of scissors in 1 mL of ice-cold Hanks-buffered salt solution and diluted with Hanks-buffered salt solution (1:40). The resulting cell suspension was subjected to alkaline single-cell gel electrophoresis. Viability was determined by the trypan blue exclusion assay.

Alkaline single-cell gel electrophoresis followed an established procedure [46], with slight modifications.

Aliquots of liver cell suspension were centrifuged (400 × *g*, 10 min). Pellets of liver cells (250 μL suspension) and blood (6 μL) samples were mixed with 65 μL low-melting agarose and put onto slides. After lysis over night (4°C, pH 10), cells were washed three times with buffer (40 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8) and treated with FPG suspension for 30 min [47]. After DNA unwinding (pH > 13, 20 min, 4°C) and electrophoresis (85 V, 300 mA, pH > 13, 20 min, 4°C), cells were washed three times with 0.4 M Tris, pH 7.4. Gels were fixed for 2 min with 95% ethanol v/v and stored at room temperature until detection. DNA was stained with ethidium bromide and viewed by computer based microscopy (Zeiss Axio Imager A1; filter set 15: excitation BP 546/12, emission LP 590; Perceptive Instruments Comet Assay 4). For each animal and organ, two slides were prepared. From each slide 2 × 50 images were scored. DNA migration was expressed as change of tail intensity (TI) *versus* negative control.

2.4.8 Statistics

Statistic analysis of data was performed by using an unpaired two-sample Student's *t*-test.

3 Results

Biomarker response after challenging two animals with 100 μg AA/kg b.w. by gavage was not found influenced by isoflurane anaesthesia. The Hb-adduct levels of the anesthetized animals were 93 ± 11 pmol/g Hb for AAVal and 36 ± 7 pmol/g Hb for GAVal, respectively, and the excreted amounts of AAMA and GAMA were 28.6 ± 0.5 and 14.4 ± 1.3% of the administered dose. The corresponding biomarker formation in the group not treated with isoflurane was 92 ± 2 pmol/g Hb for AAVal, 42 ± 10 pmol/g Hb for GAVal, 26.1 ± 2.5% for AAMA and 15.2 ± 0.4% for GAMA.

3.1 AA- and GAVal-adduct formation in Hb

In the feeding study with repeated dietary doses of 50 and 100 μg AA/kg b.w., AAVal adduct levels increased about linearly with the cumulative AA uptake in foods and in drinking water (Fig. 1). In the negative control group, AAVal levels in the range of 8–23 pmol/g Hb were consistently detected, reflecting AA background exposure. The graphs showing kinetics of incremental AAVal Hb adduct build up after ingestion of AA in French fries were nearly identical to the groups receiving AA in drinking water (AA-DW). This demonstrates that the texture of sliced and reconstituted French fries had no influence on AA bioavailability as compared with uptake *via* water by gavage. Essentially similar results were obtained for the AAVal Hb adduct buildup in groups fed GB (day 1: 20 ± 4 pmol/g Hb; day 9: 359 ± 14 pmol/g Hb; data not shown in detail). Only in the BC group, AAVal Hb buildup was found slightly diminished (by 17%), as compared with the AA uptake by gavage in drinking water (Fig. 2).

In contrast to the continuous buildup of AAVal levels with increasing cumulative dose, GAVal levels (28 ± 10 pmol/g Hb) did not exceed the background level as measured in negative controls (28 ± 9 pmol/g Hb), receiving just drinking water. In a pilot study carried out prior to the main study, animals were gavaged with single doses of 0.45, 0.9 and 10 mg/kg b.w. of AA (Fig. 3). Hb-Val-adduct levels at 9 × 50 μg/kg b.w. and after an equivalent dose (450 μg/kg) given as a single shot were about identical. At 9 × 100 μg/kg about 250 pmol AAVal/g Hb was measured, whereas at 1 × 900 μg/kg about 360 pmol AAVal/g Hb was reached. It is worth noting that irrespective of whether

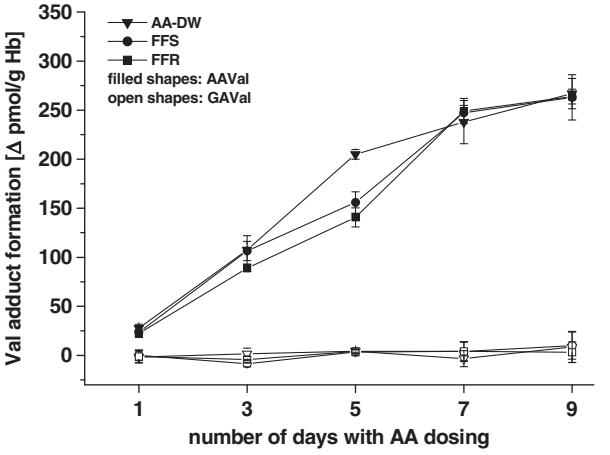

Figure 1. AA-(AAVal) and GAVal formation in Hb of SD rats 24 h after the administration of AA over 1, 3, 5, 7 and 9 days *via* French fries (FFS, FFR) and drinking water (AA-DW; 100 μg/kg b.w./day).

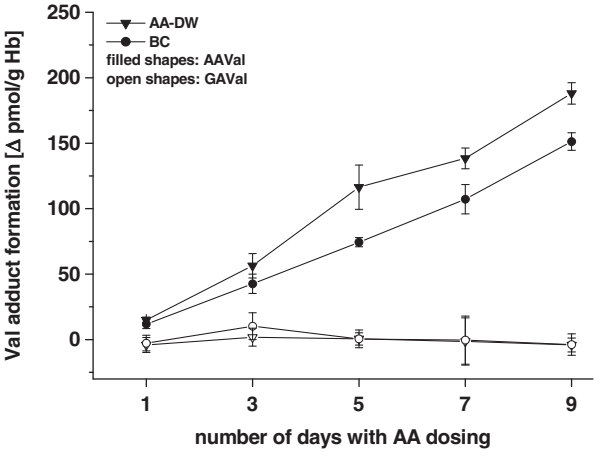

Figure 2. AA-(AAVal) and GAVal formation in Hb of SD rats 24 h after the administration of AA over 1, 3, 5, 7 and 9 days *via* BC and drinking water (AA-DW; 50 μg/kg b.w./day).

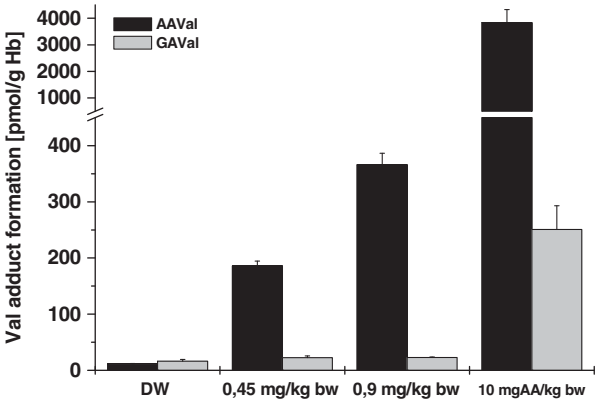

Figure 3. Formation of AA-(AAVal) and GAVal in Hb of rats 24 h after the administration of high AA doses *via* drinking water (gavage); data shown represent mean ± SD of Hb adducts averaged over three animals (10 mg AA/kg b.w., two animals).

AA was given in fractionated dosage or as single shot, no significant deviation of Hb GAVal adduct levels from background levels was observed. Only at the highest single dose applied in the pilot experiment (10 mg/kg b.w.), a significantly increased Hb GAVal adduct level (251 ± 42 pmol/g Hb) was measured (Fig. 3). In the negative control (drinking water only), GAVal levels were found slightly higher than AAVal levels. A similar observation was also reported earlier by Paulsson *et al.* and Tareke *et al.* [39, 48].

3.2 MAs of AA and GA in urine

After all time periods, significant increases in the excretion (24 h urine) of MAs from AA (50 μg AA/kg b.w.: 76 ± 25 nmol; 100 μg AA/kg b.w.: 139 ± 48 nmol) and GA (50 μg AA/kg b.w.: 34 ± 5 nmol; 100 μg AA/kg b.w.: 72 ± 17 nmol) were observed in the AA treatment groups as compared with the untreated controls (AAMA, 4–8 nmol; GAMA, not detectable). Each treatment interval resulted in very similar amounts of AAMA (35.5 ± 9.9% of dose) and GAMA (17.7 ± 3.5% of dose) throughout the repeated AA applications, independent of the number of repeated AA doses (Table 1). There was no indication for a cumulation effect. AA uptake by gavage in drinking water (AA-DW) resulted in an about 30% lower GAMA/AAMA ratio as compared with groups receiving AA in food.

GAMA/AAMA ratios were found highest after the first dosing of AA *via* drinking water or food, reflecting a somewhat lower AAMA and a higher GAMA excretion, as compared with the values observed after repeated dosing up to 9 days (Table 2). AAMA excretion in the BC group was found about 20% lower as compared with the corresponding AA-DW group, indicative for some reduction in bioavailability from BC. In contrast to BC, AA uptake in the French fries groups resulted in biomarker responses very similar to those observed after ingestion of AA *via* drinking water. Taken all treatment groups together, 53% of the ingested daily dose were recovered as MAs in urine.

3.3 AA and GA in urine

Minor proportions (1.1–5.6%) of AA were recovered from urine, together with traces of GA (0.6–3.5%), thus only marginally contributing to the total AA and AA metabolite excretion balance (Table 1). Urinary AA in the groups receiving AA in drinking water (AA-DW) was found two to threefold higher as compared with the corresponding food groups. AA excretion was lower after single dosing of AA *via* drinking water or food, resulting in a markedly higher initial GA/AA ratio as compared with up to ninefold repeated dosing over up to 9 days (Table 2).

3.4 AA and GA in feces

Weight of feces samples collected over 24 h after the last daily dose ranged between 4 and 18 g. Significant variability in fecal AA excretion within individual animals of the respective treatment groups was observed. AA amounts in feces accounted for 2.0 ± 2.4% (0.4−8.2%; median: 1.2%) of the total dose (data not shown). In 37% of the samples, the AA contents were below the LOD. GA was not detectable in any sample. In total, no significant differences between all groups were observed and fecal excretion was found to only marginally contribute to the overall balance.

3.5 Kinetics of AA and GA serum levels

Kinetics of AA and GA plasma concentrations after single dosing in water and in French fries was investigated within a time period between 30 min and 4 h after dosing (Fig. 4). Peak plasma concentrations (c_{max}) of AA-DW: 1.84 ± 0.17 μM, FFS: 0.44 ± 0.05 μM were already measured at the first time point investigated (30 min). However, about fourfold higher levels were reached in the group, receiving AA in water (AA-DW) as compared with the groups receiving the same dose in French fries (FFS). During the observation period, AA levels in the AA-DW group decreased with an apparent half life of roughly 3 h, whereas in the FFS group they reached a plateau, reflecting delayed liberation from food during gastrointestinal passage and resulting in much longer persistence, whereas AA taken up in water was rapidly absorbed and eliminated. Traces of GA (< 0.06 μM) were detectable in both treatment groups only 4 h after AA administration (data not shown).

3.6 DNA damage in leukocytes and hepatocytes

TI values of exposed groups differed from untreated controls by not more than about ± 5%. Also, the TI values of animals

Table 1. AA, GA, AAMA, GAMA in percentage of dose excreted in urine during 24 h after last dosing

	Days	AA	GA	GA/AA	AAMA	GAMA	GAMA/AAMA	ΣMA
AA-DW 100 μg/kg b.w.	1	2.4 (0.5)	3.4 (0.2)	1.45 (0.33)	26.0 (3.3)	20.1 (2.5)	0.77 (0.03)	46.1 (5.7)
	3	6.4 (1.9)	1.9 (0.2)	0.32 (0.07)	30.3 (0.2)	9.6 (1.0)	0.32 (0.03)	39.9 (1.1)
	5	5.3 (0.6)	2.8 (0.3)	0.53 (0.09)	52.1 (2.0)	20.4 (1.1)	0.39 (0.04)	72.5 (0.9)
	7	8.4 (3.1)	1.5 (0.2)	0.20 (0.09)	31.2 (2.7)	14.7 (2.2)	0.47 (0.08)	46.0 (3.5)
	9	5.7 (3.7)	1.9 (0.3)	0.43 (0.16)	38.6 (2.0)	12.3 (0.2)	0.32 (0.02)	50.9 (2.0)
	Mean	5.6 (3.0)	2.3 (0.7)	0.59 (0.48)	35.7 (9.5)	15.4 (4.6)	0.46 (0.17)	51.1 (11.7)
FFS 100 μg/kg b.w.	1	2.5 (0.3)	3.1 (0.1)	1.27 (0.15)	23.6 (1.7)	25.9 (1.8)	*1.10 (0.02)**	49.4 (3.5)
	3	*1.6 (0.8)*♯	1.7 (0.1)	1.74 (1.42)	*30.8 (5.7)*♯	*22.5 (4.6)**	*0.73 (0.01)**	53.2 (10.1)
	5	*2.2 (0.3)*♯	2.2 (0.6)	1.01 (0.31)	38.0 (2.0)	19.4 (1.3)	0.51 (0.06)	*57.4 (1.7)*♯
	7	*1.7 (0.7)*♯	1.3 (0.5)	*0.84 (0.08)**	26.1 (7.7)	15.4 (3.5)	0.61 (0.09)	41.4 (10.9)
	9	2.2 (0.2)	1.9 (0.5)	0.88 (0.20)	*31.8 (1.2)*♯	*19.7 (1.3)**	*0.62 (0.05)**	51.5 (1.4)
	Mean	*2.0 (0.6)*	2.0 (0.7)	*1.15 (0.74)*	30.0 (6.7)	*20.6 (4.5)*	*0.72 (0.21)*	50.6 (8.7)
FFR 100 μg/kg b.w.	1	1.6 (0.2)	*2.4 (0.3)*♯	1.47 (0.04)	22.0 (2.0)	23.4 (3.4)	*1.06 (0.06)**	45.3 (5.4)
	3	*1.1 (0.5)*♯	*1.0 (0.4)*♯	*0.90 (0.14)**	*20.5 (3.7)*♯	12.8 (3.3)	*0.61 (0.06)**	33.2 (7.0)
	5	*1.7 (0.3)*♯	*1.8 (0.3)*♯	1.12 (0.41)	*39.6 (3.1)*♯	*17.3 (1.0)*♯	0.44 (0.06)	*56.9 (2.4)*♯
	7	*1.4 (0.1)*♯	1.2 (0.1)	*0.91 (0.17)**	*40.1 (2.9)**	16.3 (1.0)	0.41 (0.04)	*56.4 (2.9)**
	9	1.7 (0.4)	1.9 (0.3)	*1.16 (0.21)**	*28.0 (3.5)*♯	20.8 (1.2)*	*0.76 (0.14)**	48.8 (2.8)
	Mean	*1.5 (0.4)*	*1.7 (0.6)*	*1.11 (0.31)*	30.0 (9.0)	18.1 (4.3)	*0.66 (0.25)*	48.1 (9.7)
GB 100 μg/kg b.w.	1	1.7 (0.2)	2.3 (0.7)	1.32 (0.36)	21.2 (1.3)	21.0 (0.6)	*0.99 (0.08)**	42.2 (1.0)
	3	*1.1 (0.2)*♯	1.5 (0.2)	*1,41 (0.08)**	*45.8 (1.7)**	*13.8 (0.4)**	0.30 (0.02)	*59.5 (1.6)**
	5	*1.8 (0.3)*♯	2.4 (0.6)	1.38 (0.45)	*31.1 (2.2)*♯	*15.1 (0.9)*♯	0.49 (0.03)	*46.2 (2.8)*♯
	7	*2.0 (0.3)*♯	1.4 (0.2)	*0.68 (0.04)**	*55.5 (3.4)**	21.9 (3.2)	0.41 (0.01)	*77.4 (6.6)**
	9	2.5 (0.2)	1.4 (0.1)	0.58 (0.06)	*48.5 (1.8)**	*17.6 (0.5)**	0.36 (0.01)	*66.0 (2.1)**
	Mean	*1.8 (0.5)*	1.8 (0.6)	*1.07 (0.45)*	40.4 (12.6)	17.8 (3.5)	0.51 (0.25)	58.3 (13.3)
AA-DW 50 μg/kg b.w.	1	1.4 (0.1)	2.0 (0.4)	1.40 (0.20)	23.2 (3.2)	17.0 (2.7)	0.73 (0.04)	40.2 (5.8)
	3	4.5 (1.3)	2.6 (0.3)	0.61 (0.15)	50.4 (3.2)	16.0 (1.8)	0.32 (0.06)	66.4 (1.4)
	5	4.7 (1.2)	3.4 (0.5)	0.82 (0.35)	40.1 (3.7)	17.0 (3.5)	0.43 (0.11)	57.1 (4.3)
	7	5.7 (1.4)	6.7 (3.4)	1.08 (0.37)	53.7 (8.6)	12.6 (0.9)	0.24 (0.03)	66.3 (9.5)
	9	8.6 (3.3)	2.6 (0.6)	0.42 (0.31)	44.6 (1.9)	17.9 (2.9)	0.40 (0.08)	62.6 (1.7)
	Mean	5.0 (2.9)	3.5 (2.3)	0.87 (0.45)	42.4 (11.7)	16.1 (3.2)	0.43 (0.18)	58.5 (11.2)
BC 50 μg/kg b.w.	1	2.0 (0.3)	3.2 (1.2)	1.56 (0.44)	20.9 (4.4)	20.0 (3.2)	0.98 (0.15)	40.9 (7.0)
	3	2.9 (0.6)	2.3 (0.2)	0.84 (0.19)	*36.1 (4.9)*♯	15.0 (1.6)	0.43 (0.08)	*51.1 (4.6)*♯
	5	2.8 (0.2)	3.3 (0.3)	1.19 (0.18)	31.3 (3.7)	16.3 (1.2)	0.53 (0.09)	47.5 (3.1)
	7	3.2 (0.3)	3.1 (0.2)	0.97 (0.15)	*34.4 (1.7)*♯	15.9 (1.6)	*0.46 (0.04)**	50.3 (2.9)
	9	3.0 (0.2)	2.4 (0.2)	0.81 (0.10)	36.8 (4.0)	18.1 (0.1)	0.50 (0.05)	54.9 (4.1)
	Mean	*2.8 (0.5)*	2.9 (0.7)	1.07 (0.37)	*31.9 (7.0)*	17.0 (2.5)	*0.58 (0.22)*	*48.9 (6.5)*
AA- DW 450 μg/kg b.w.	Single dose	3.5 (1.5)	2.7 (0.1)	0.99 (0.51)	43.1 (2.2)	17.6 (1.7)	0.41 (0.06)	60.7 (0.5)
AA-DW 900 μg/kg b.w.	Single dose	5.2 (1.6)	2.6 (0.2)	0.54 (0.12)	39.4 (3.3)	17.1 (0.5)	0.43 (0.03)	56.5 (3.5)
AA-DW 10 mg/kg b.w.	Single dose	1.1 (0.5)	0.6 (0.2)	0.53 (0.03)	35.1 (0.6)	22.6 (1.5)	0.65 (0.03)	57.6 (2.2)

Mean values of three animals *per* group, 10 mg AA/kg b.w., two animals (SD). FFR, French fries made of potato products; ΣMA: sum of AAMA and GAMA; *significantly increased compared with AA-DW; ♯, significantly decreased compared with AA-DW; $p<0,05$.

Table 2. AA, GA, AAMA, GAMA contents in urine collected for 24 h after the last dosing expressed as % of the given daily dose

Days	AA	GA	GA/AA	AAMA	GAMA	GAMA/AAMA
1	1.9 (0.5)	2.7 (0.8)	1.41 (0.30)	22.8 (3.3)	21.2 (3.8)	0.94 (0.16)
3	2.9 (2.2)	*1.8 (0.6)*	*0.97 (0.76)*	*35.6 (10.7)*	*15.0 (4.7)*	*0.45 (0.17)*
5	*3.1 (1.5)*	2.6 (0.8)	*1.01 (0.42)*	*38.7 (7.6)*	*17.6 (2.5)*	*0.47 (0.09)*
7	*3.7 (2.9)*	2.5 (2.4)	*0.78 (0.34)*	*40.2 (12.2)*	*16.1 (3.6)*	*0.43 (0.13)*
9	*4.0 (3.2)*	*2.0 (0.5)*	*0.71 (0.33)*	*38.1 (7.5)*	*17.7 (3.0)*	*0.49 (0.17)*

Values represent mean values ($n = 18$) of a treatment period over all treatment groups (SD).

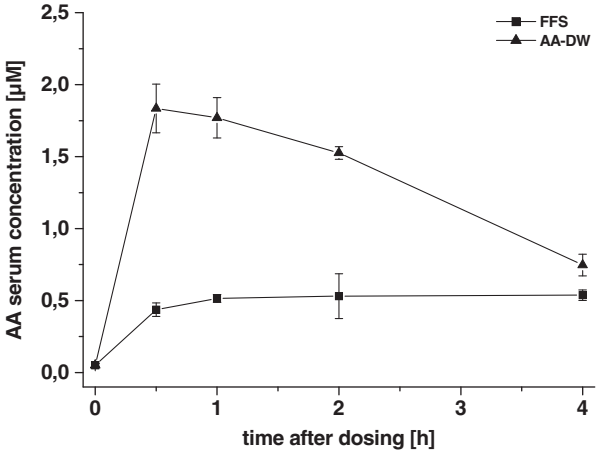

Figure 4. AA serum concentrations following a single dose of 100 µg/kg b.w. AA *via* French fries (FFS) or drinking water (AA-DW); mean ± SD; $n = 3$.

given 50 µg AA/kg b.w./day in BC or drinking water under all conditions remained within about ±2% of untreated control values (Fig. 5). However, in animals given a single dose of 10 mg AA/kg b.w. an induction of DNA damage was observed.

4 Discussion

Hb adducts at the *N*-terminal valine are established biomarkers of internal exposure with AA and its metabolite GA [25, 26, 28, 48, 49]. In this study, rats received AA *via* drinking water and in parallel *via* different food matrices containing AA in process related contents. AAVal Hb levels in all AA treatment groups increased in a nearly linear fashion with time of exposure and thus with cumulative dose in the repeated feeding/gavage study. In contrast to the AAVal levels, GAVal levels were not found to significantly increase above the background range of untreated controls. Thus, within the limits of the analytical sensitivity achieved by our method, in SD rats the biomarker depicting GA in the vascular system after uptake from the GI tract and first pass through the liver did not significantly deviate from background levels of untreated controls. The range of AAVal levels found in untreated control SD rats was 8–23 pmol/g Hb. GAVal levels were in the range of 27–33 pmol/g Hb, thus being 1.4–3.5 times higher than the levels of AA adducts. Comparable background levels in rats were found by Tareke *et al.*, with a GAVal/AAVal ratio of 2–3 and also by Paulsson *et al.*, with a ratio of 1.8 [39, 48].

AAVal/GAVal ratios reported in the literature show considerable variations. In a single-dose experiment in F344 rats gavaged with 100 µg AA/kg b.w. in water, AAVal adduct levels were reported in a similar range as found by us. [48]. However, enhanced GAVal Hb formation was observed, reaching three times the levels of the AA adducts. Similarly, higher GAVal than AAVal levels were also found by Törnqvist *et al.* after continuous application of AA in drinking water at dose levels of 0.1–2.0 mg/kg b.w./day to F344 rats, equivalent to cumulative doses of 0.7–14 mg AA/kg b.w. [50]. At the high dosage, GAVal formation was found more than tenfold higher as compared with the value of our single-dose experiment at 10 mg/kg b.w. However, AAVal levels only reached about 50% of the levels observed by us. This discrepancy may be reconciled by the fact that in our experiment, AA was applied as a bolus, thus reaching much higher c_{max} values, followed by relatively rapid clearance, as also demonstrated in our short-term toxicokinetics experiment. Concentration-driven reactions with cellular nucleophiles, in line with the mass action law, are also supposed to contribute to enhanced clearance. On the contrary, much lower, yet more persisting levels will prevail under continuous uptake through drinking water, as also observed in our continuous feeding study. This should allow for more efficient metabolic formation of GA as compared with high-dosage conditions, where AA to GA biotransformation might also become saturated. This interpretation is supported by reports on higher AAVal than GAVal in SD- and F344-rats dosed with 100 mg AA/kg b.w. [39], 50 mg AA/kg b.w. [40] and 3 mg AA/kg b.w. [25]. It is worth noting that GA, devoid of Michael reactivity is reported to be more reactive towards *N*-terminal valine than AA [39, 51].

Reactivity of AA and GA towards sulfhydryl groups is higher than to amino groups and there are multiple SH groups, especially those provided by cysteines in Hb [52]. Thus, monitoring *N*-terminal Val-adduct formation is considered to just cover a minor part of total Hb binding. Therefore, although GA became detectable in traces only at 4 h in our short-time toxicokinetics study, these might still have resulted in significant cysteine adduct formation in Hb, not reflected by GAVal. In mice, more efficiently

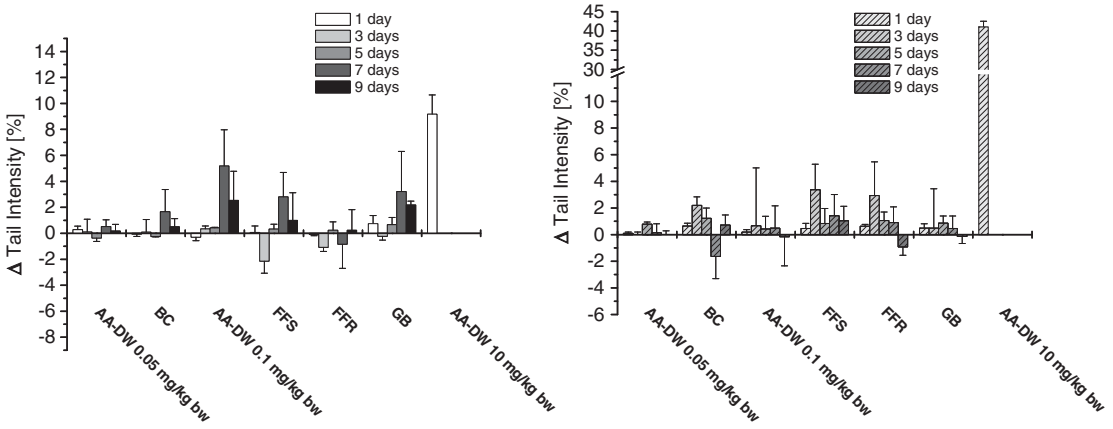

Figure 5. Change of DNA damage in hepatocytes and leukocytes of rats after 1, 3, 5, 7, and 9 days administration of 100 μg AA/kg b.w./day *via* French fries (FFS, sliced from potatoes; FFR, made of potato products), GB) and drinking water (AA-DW, 0.1 mg/kg b.w.), 50 μg/kg b.w./day *via* BC and drinking water (AA-DW, 0.05 mg/kg b.w.) and 10 mg AA/kg b.w. (AA-DW, 10 mg/kg b.w.) as compared with negative control; (open bars, hepatocytes; striped bars, leukocytes; mean ± SD, three animals *per* group, AA-DW, 10 mg/kg b.w., two animals).

metabolizing AA to GA as compared with rats and humans, significant GAVal adduct levels appeared in the blood after a dose up to 58 μg AA/kg b.w. [41].

As a measure for overall bioavailability of AA from drinking water and the various foods investigated, incremental AAVal Hb adduct buildup over time followed very similar kinetics. Only with BC, a slight reduction (up to 20%) of AAVal formation became apparent. Within the accuracy of the experiment, AAVal formation with all other food was not significantly different from application *via* drinking water. Moreover, total excretion of MAs was very similar when comparing AA uptake from French fries and GB with the uptake from water by gavage. Total MA excretion in the BC group in most cases was found slightly decreased. These results indicate that total uptake of food-borne AA and internal dose is not substantially influenced by the food matrix.

The total amount of AA-related compounds excreted *via* urine was in the range of 35–81% of the administered dose (mean, 58.6 ± 10.8%; median, 56.6%, Table 1). This shows that a considerable proportion of the applied dose remains unaccounted for in the metabolic balance. The high electrophilic reactivity of AA may result in covalent binding to a spectrum of nucleophiles in bio(macro)molecules, including amino, hydroxyl, thiol and other groups [53]. Also, glyceramide, not determined within the scope of this study, might be another minor metabolite of AA found in rat urine after administration of 50 but not after 3 mg of $^{13}C_3$-AA/kg b.w. [25, 31]. However, a different situation appears to prevail in humans found to excrete significant amounts of glyceramide at the same dose level [25].

Our results correspond to the observations of Bjellaas *et al.*, who compared subcutaneous application of AA with its oral uptake *via* feeding of crisp bread and found excretion of AA metabolites in urine practically identical, indicating a nearly complete uptake of AA from food [33]. On the contrary, a reduced AA uptake has been reported by Doerge *et al.*, comparing serum area under the curves after uptake of 100 μg AA/kg b.w. given i.v., in drinking water (gavage) or in diet fortified with AA [6]. In comparison to i.v. administration, bioavailability after gavage was calculated in that study to be 60–98% and from fortified diet only 32–44%. Total urinary metabolite contents (sum of AA, GA, AAMA and GAMA) in the AA fortified food group were 45% compared with gavage [54]. It should be borne in mind, however, that the high reactivity of AA towards nucleophilic food components [55, 56] when added to food might be responsible for such an apparently reduced bioavailability.

High AA absorption from the GI tract is supported by our observation that AA excreted *via* feces in all AA treatment groups only marginally contributes to the total AA and AA metabolite excretion. A similar excretion rate *via* feces has been reported earlier after dosing rats with ^{14}C-labelled AA [5]. Amounts of AA excreted in feces of BC groups were as low as for the other feeding groups and give no evidence for an enhanced excretion of AA *via* feces. GA concentrations in feces were below the LOD, indicating that under the conditions tested, the gut flora of rats does not appear to significantly convert AA to GA.

MA excretion in urine collected 24 h after dosing reflects total glutathione adduct formation and its further processing to MA. Generally, a mean of 53% of a given AA dose was detoxified and eliminated *via* MA excretion, in line with the earlier findings from the literature [7, 34]. Assuming similar formation kinetics for AA and GA glutathione adducts in the liver, urinary GAMA levels indicate about 20% of a given AA dose to be metabolized to GA in SD rats. In the single high-dose experiment with 0.45, 0.90 and 10 mg AA/kg b.w., the two lower doses reflect the respective cumulative doses reached in the repeat dose experiments. In these two lower dosages, percentages of total MA excretion (24 h) were similar to those of the animals receiving 50 or 100 μg AA/kg b.w. in nine consecutive applications, respectively.

It is worth noting that significantly higher levels of GAMA and concomitantly lower levels of AAMA were excreted in the single-dose groups (day 1; 50 or 100 μg AA/kg b.w.). This may indicate some adaptive response towards detoxification by MA formation in animals receiving repeated doses (up to day 9; 50 or 100 μg AA/kg b.w.).

In contrast to the total AA uptake over time, significant differences in absorption kinetics between AA uptake over drinking water (AA-DW) and French fries (FFS) were observed in the single-dose short-term toxicokinetics experiment, using AA serum levels as biomarker. The maximal AA serum concentration after 30 min corresponded to fourfold higher levels in the AA-DW group as compared with the FFS group. Similarly, Doerge *et al.* observed higher AA serum peak concentrations 2 h after administration of AA by gavage compared with AA uptake over fortified diet [6]. These differences in absorption kinetics indicate that AA-uptake from DW is significantly faster as compared with its uptake from French fries, reflecting delayed AA liberation and/or absorption from food in the upper gastrointestinal tract.

The latter observation might also explain differences in GAMA/AAMA ratios observed for AA uptake in French fries (FFS), reaching 0.72 ± 0.21, whereas for its uptake in drinking water only 0.46 ± 0.17 was found. This appears to indicate that hepatic phase I metabolism of AA to GA might be more efficient at delayed AA uptake, consistent with slower liberation from the food matrix. By the same token, higher AA excretion rates in the AA-DW groups might reflect the higher AA peak plasma levels reached, as discussed.

In the satellite experiment with a single dose of 100 μg AA/kg b.w., applied by gavage, GA became just barely detectable at a single time point, with 8- to 12-fold lower plasma concentration than AA (< 0.06 μM, 4 h). In contrast to these results, Doerge *et al.* found peak GA concentrations of 0.2 μM in plasma already 2 h after administration of 100 μg AA/kg b.w., given to male F344 rats *via* gavage or fortified diet [6]. Furthermore, Doerge *et al.* reported higher contents of GA and GAMA in urine (6%, respectively, 27–29% of the dose applied), at variance to our results [54]. These marked differences may at least in part be explained by differential phase 1 metabolic turnover of AA to GA, as reported by Tareke when comparing metabolic capacity of liver microsomes from SD rats and F344 rats [48]. Also, differential tumour induction was observed between rats and mice [57, 58]. In the same dose range, thyroid tumours were found only in rats, not in mice.

In humans, several studies have shown that at best 6% of an ingested dose account for the formation of GA, GAMA and glyceramide [25, 30, 43]. Altogether, these data suggest broad intra and interspecies differences concerning metabolism of AA to GA. Humans apparently are relatively poor metabolizers in terms of conversion of AA into GA and more efficient with respect to MA formation, as also discussed earlier by Fennel *et al.* and Fuhr *et al.*[25, 30]. In the comet assay, only at the high dose level of 10 mg AA/kg b.w. significant induction of DNA damage was observed after treatment with FPG. The comet assay has limited sensitivity, though. In our previous *in vitro* studies, significant induction of DNA damage became only detectable by comet assay in blood cells after 4 h incubation of blood with 10 μM GA, in connection with DNA post-treatment processing with formamidopyrimidine glycosylase (FPG). FPG treatment introduces strand breaks at abasic sites, oxidative DNA lesions and *N*7-guanine alkylation sites [15]. It was demonstrated that these lesions rapidly disappeared within 8 h after GA treatment. The lowest concentration found to be active in this system (10 μM) is more than 100-fold higher than that reached after single-dose application of 100 μg AA/kg b.w. in our short-term toxicokinetics experiment.

Our data indicate that within the dose range of at least up to 100 μg AA/kg b.w., GA formed in the liver is effectively coupled to GSH. Whether extra hepatic metabolism of AA and/or its reactivity with structural or plasma proteins also contribute to its biological effectiveness and clearance is not covered by the present investigation.

Our results were obtained in male SD rats. Findings by several groups indicate that humans are less proficient than rodents in activating AA metabolically to GA [25, 34, 54] and may be more proficient in detoxification reactions such as coupling to glutathione [30].

AA is ranked as genotoxic carcinogen and has been allocated an MOE of < 1000 [2, 3]. The results of our study demonstrate that more mechanism-based research is needed to better assess and understand dose–response effects in the low dose range corresponding to dietary intake, to achieve science-based risk assessment. This is especially true with respect to kinetics of activating and detoxifying biotransformations and their impact on the biological outcome at low dosage. Such research also needs to include advanced physiologically based biokinetic modelling, together with the best-available methodology to approach, as closely as possible, human intake levels.

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