Cancer Risk Assessment for the Environmental Mutagen and Carcinogen Crotonaldehyde on the Basis of TD$_{50}$ and Comparison with 1,N$_2$-Propanodeoxyguanosine Adduct Levels$^1$

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Abstract
Humans are ubiquitously exposed to crotonaldehyde to a strongly varying extent, in particular, via food and alcoholic beverages. Like other $\alpha$,$\beta$-unsaturated carbonyl compounds, crotonaldehyde forms 1,N$_2$-propanodeoxyguanosine adducts and is genotoxic, mutagenic, and carcinogenic. This study was designed to perform a cancer risk assessment on the basis of TD$_{50}$ which was available from a long-term cancer study with F-344 rats (F. L. Chung et al., Cancer Res., 46: 1285–1289, 1986), and the estimated daily intake via food and beverages. A relatively high cancer risk of 0.1–1 cancer incidence/10$^3$ humans was extrapolated on the basis of the TD$_{50}$ from the cancer study of Chung et al. for the estimated dietary intake and drinking wine. To compare the 1,N$_2$-propanodeoxyguanosine DNA adduct levels of crotonaldehyde with the assessed cancer risk, we synthesized adduct standards and developed a $^{32}$P-postlabeling method for DNA adducts of crotonaldehyde providing a detection limit of 3 adducts/10$^9$ nucleotides. Repeated gavages of 10 and 1 mg/kg were given to simulate the steady-state situation of the animal cancer study of Chung et al. and to estimate the adduct levels after intake of crotonaldehyde via food. The estimated adduct levels at these crotonaldehyde intakes were in the range of 3 adducts/10$^9$ nucleotides. The adducts persisted to a certain extent. The persistence is important for considering the steady-state situation after permanent intakes of crotonaldehyde via food. However, the adducts are repaired to some extent; 2 weeks after the last of repeated gavages, only 19% of the initial amount measured directly after the last gavage is left. According to our results, a steady-state concentration in the range of 3 adducts/10$^9$ nucleotides is responsible for the induction of cancer in the study of Chung et al., in the case that cancer from crotonaldehyde depends exclusively on the 1,N$_2$-propanodeoxyguanosine adducts considered here. No propanodeoxyguanosine adducts of crotonaldehyde were found in the DNA of untreated animals in our studies.

Introduction
Crotonaldehyde is an important environmental pollutant. It is formed during combustion of carbon-containing fuels and other materials. Lipari et al. (1), for instance, calculated an emission of 140–2700 metric tons of crotonaldehyde per year in the United States due to burning wood in fireplaces, based on the consumption of firewood. Concentrations of 0.02–17 mg/m$^3$ were measured in automobile exhausts (2, 3); however, surprisingly low concentrations of crotonaldehyde in the range of 1.1–2.1 µg/m$^3$ were found near highways at a distance of 1 m (4). Another important factor is cigarette smoking. Relatively high amounts of 72–228 µg of crotonaldehyde are formed per smoked cigarette (5, 6). Crotonaldehyde is evidently also formed during biological degradation of organic material, e.g., plants. In exhausts of house garbage compost plants, amounts of 2.9 mg/m$^3$ were measured. Strongly varying concentrations of crotonaldehyde are reported to occur in food, e.g., in fish [71–1000 µg/kg (7, 8)], in meat [10–270 µg/kg (9, 10)], and in fruits and vegetables [1–100 µg/kg (11, 12)]. Crotonaldehyde was also found in alcoholic beverages like wine (0.3–1.24 mg/liter; Ref. 13) or whisky (30–210 µg/liter; Ref. 14). Crotonaldehyde is an important industrial chemical [e.g., for the synthesis of tocopherol (vitamin E), the food preservative sorbic acid, and the solvent 3-methylbutanol] but is also a contaminant and byproduct in various chemical processes. In the meantime, crotonaldehyde is considered as “justifiably suspected of having carcinogenic potential” in the German “MAK” list, and there is no threshold limit value at the work place; however, in 1987–1991, crotonaldehyde concentrations of 0.3–1.5 mg/m$^3$ were measured at certain work places$^1$ in the chemical industry.

The data indicate that humans are permanently exposed to this compound via different routes and to a strongly varying extent.

Crotonaldehyde is genotoxic (15–17), mutagenic (18–20), and carcinogenic (21, 22), and it forms exocyclic 1,N$_2$-propanodeoxyguanosine adducts as the main DNA adducts (23, 24).

All data available suggest that crotonaldehyde can play a significant role in carcinogenesis; however, the data do not allow a clear cancer risk assessment of crotonaldehyde. Furthermore, contradictory results were reported on the background of crotonaldehyde DNA adducts in the organs of untreated Fischer 344 rats. Whereas we could not find 1,N$_2$-
propanodeoxyguanosine adducts according to our detection limit of 3 adducts/10⁶ nucleotides (25, 26). Nath and Chung (27) found adducts in untreated Fischer 344 rats. This discrepancy does not necessarily depend on the methodological differences in the ³²P-postlabeling procedures applied in both groups but can also be explained by differences in crotonaldehyde exposures of the animals. As demonstrated above, strongly varying exposures were reported for humans in the literature, and this is, in principle, also valid for experimental animals.

This study was designed to give an approach to a tentative cancer risk assessment of crotonaldehyde on the basis of the T₉₀ of crotonaldehyde and the daily intake of crotonaldehyde via food. To investigate a possible relationship between cancer risk and DNA adduct levels, we simulated the steady-state dose conditions of the cancer study of Chung et al. (21) and measured the ¹N₂-propanodeoxyguanosine DNA adduct levels in Fischer 344 rats after multiple gavage of doses of 1 and 10 mg of crotonaldehyde for 4 weeks.

Materials and Methods

Chemicals and Reagents. Crotonaldehyde is irritating, genotoxic, mutagenic, and carcinogenic. trans-Crotonaldehyde ([E]-2-butenal, “Gold Label” boiling point, 104°C/760 Torr) was purchased from Aldrich (Steinheim, Germany). dGp,² apyrase from potato, RNase T₁, micrococcus nuclease from Staphylococcus aureus, and NP₁ from Penicillium citrinum were obtained from Sigma Chemical Co. (Deisenhofen, Germany). RNase A was from Serva (Heidelberg, Germany), proteinase K was from Merck (Darmstadt, Germany), and spleen phosphodiesterase was from Boehringer (Mannheim, Germany). [γ-[³²P]ATP and T₄ PNK were obtained from Amersham (Braunschweig, Germany). PEI-cellulose TLC plates were from Macherey & Nagel ( Düren, Germany). All other reagents were bought from the Sigma Chemical Co.-Altrich Chemie GmbH group (Deisenhofen, Germany), Merck, Serva, or Appligene-Oncor and were of the best quality available.

Synthesis of Crotonaldehyde Adducts of dGp (Standards).

The standards were synthesized as described previously (26) by reacting 80 mg (0.21 mmol) of dGp and 126 mg (1.8 mmol) of crotonaldehyde in 40 ml of a 100 mM phosphate buffer (pH 8.5) at 80°C. The adduct standards were isolated by high-performance liquid chromatography as described previously (26) with Waters system Millenium 2010 software, Waters Model 510 pumps, Waters 486 UV detector, Rheodyne 7125. Knauer reverse-phase 18 column (length, 300 mm; inside diameter, 8 mm), and a linear gradient from 10 mM ammonium formate buffer (pH 4.7) to methanol with a flow rate of 4 ml/min in 40 min at 360 nm. A maximum yield of 30% was obtained after 20 h at 80°C and pH 8.5. The chemical structure of the two diastereomers of the adduct standards isolated under the high-performance liquid chromatography conditions is shown in Fig. 1.

The UV spectra were recorded at different pH values: (a) UV (pH 1), λₘₐₓ = 262 nm and λₘᵢₙ = 231 nm; (b) UV (pH 7), λₘₐₓ = 258 nm and λₘᵢₙ = 229 nm; and (c) UV (pH 13), λₘₐₓ = 260 nm and λₘᵢₙ = 229 nm.

¹H nuclear magnetic resonance (Bruker 600 MHz, D₂O): δ 1.2 (d, J₁, J₂ = 6.3 Hz, [H₁H₁]), 1.6 (m, J₉,₄ = 13.0 Hz, 1H, H-7a), 2.2 (pseudo-t, J₉,₄ = 13.0 Hz, J₆,₇ = 13 Hz, J₇,₈ = 2.1), 3.3 (H₂)z, 1H, H-7b), 2.6 (dd, J₉,₄ = 13.8 Hz, J₇,₈ = 6.3 Hz, J₆,₇ = 3.1 Hz), 1H, H-2a), 2.8 (pseudo-t, J₉,₄ = 13.0 Hz, J₆,₇ = 7.0 Hz, J₇,₈ = 7.0 Hz, J₈,₉ = 2), 3.7 (m, H-1, H-6), 3.75 (dd, J₇,₈ = 12.0 Hz, J₈,₉ = 4.7 Hz, H-5a), 3.8 (dd, J₉,₈ = 12.4 Hz, J₈,₉ = 3.4 Hz, H-5b), 4.2 (pseudo-β, J₉,₈ = 3.6 Hz, J₈,₉ = 3.6 Hz, H-1H-4), 6.25 (pseudo-β, J₉,₈ = 7.0 Hz, J₈,₉ = 7.0 Hz, H-1H-4), 6.28 (pseudo-β, J₉,₈ = 2.6 Hz, J₈,₉ = 2.6 Hz, H-1, H-8), 7.9 (s, H-1, H-2).

¹C nuclear magnetic resonance (Bruker 150.9 MHz, D₂O): δ(ppm) 22.2 (C-11), 37.9 (C-7), 39.9 (C-2'), 42.0 (C-6), 64.0 (C-5'), 73.2 (C-8), 77 (C-3'), 87 (C-1), 89 (C-4'), 110 (C-10b), 140 (C-2), 153 (C-3b), 154 (C-4b), 161 (C-10).

Mass spectrometry electrospray (70 keV) (Finnigan MAT-Triò 2000 quadrupole): m/z 439 (70 Na¹), 220 (M-D₄+H⁺).

DNA Binding Studies in Vivo. Oral doses of either 10 mg/kg body weight in 0.1 ml of corn oil or 1 mg/kg body weight in 0.1 ml of corn oil were administered to 8-week-old female Fischer 344 rats five times a week for 6 weeks. The rats were sacrificed 20 h after the last gavage. Four rats were used for each group, and two determinations were carried out.

Persistence of the Adducts. Oral doses of 10 mg/kg body weight were administered to 8-week-old female Fischer 344 rats purchased from Harlan Winkelmann (Borchen, Germany) 5 days a week for 4 weeks. The first group of animals was sacrificed 24 h after the last gavage (i.e., 4 weeks and 1 day after the start of the application), the second group was sacrificed 1 week after the last gavage (5 weeks after the start of the application), and the third group was sacrificed 2 weeks after the last gavage (6 weeks after start of the application).

Isolation of DNA. The standard method described by Gupta (28) was found to provide the best results and was used throughout these investigations.

DNA Hydrolysis. DNA (10 μg) was incubated for 4 h at 37°C with 5 μl of enzyme mixture containing 0.2 unit/μl (1 μg/μl) micrococcus nuclease and 0.002 unit/μl (1 μg/μl) spleen phosphodiesterase and with 4 μl of DNA digestion buffer [25 μM CaCl₂, 50 μM sodium succinate (pH 6.0)] in a total volume of 20 μl.

NP₁ Treatment. Six μl of NP₁ mixture consisting of 1.2 μl (6 μg) of NP₁ solution, 1.8 μl ZnCl₂, and 3 μl of 250 μM sodium acetate (pH 4) were added to a volume of 16.3 μl of digested DNA (10 μg). The mixture was incubated for 1 h at 37°C, and the reaction was stopped by adding 2.4 μl of 0.5 M Tris base.

*The abbreviations used are: dGp, deoxyguanosine 3'-monophosphate; LE, labeling efficiency; NP₁, nuclease P1; PEI, polyethylene imine; PNK, polynucleotide kinase; RAL, relative adduct labeling.
The solution was desiccated to dryness and redissolved in 10 μl of water.

**³²P Postlabeling.** Labeling mixture (8.3 μl) was added to the digested and enriched solution. The labeling mixture consisted of 4 μl of kinase buffer [200 mM bicine/NaOH (pH 8.7), 100 mM DTT, 10 mM spermidine, and 25 mM MgCl₂], 4 μl of [γ-³²P]ATP (167 TBq/4500 Ci/mmol), and 0.25 μl (7.5 units) of T4 PNK. The sample was incubated at 37°C for 55 min. Then, 4 μl (40 milliunits) of apyrase were added, and the solution was incubated for 40 min at 37°C. With this method, a LE of 80–90% was achieved. The determination of the LE and the dependence of the LE on different parameters such as incubation time, amount of [γ-³²P]ATP, pH value, and amount of PNK were recently described and discussed in detail (26).

**PEI-cellulose TLC.** A 3.5-cm wick (Whatman 17) was attached to a 13.5 × 20-cm prewashed PEI-cellulose sheet from Macherey & Nagel. The eluent in the first dimension was 0.7 M ammonium formate (pH 5.5). After a development of 3.5 h, the wick was cut off, the sheet was dried, and another 3-cm wick (Whatman 1) was attached in the second dimension. The eluent in the second dimension was 0.3 M ammonium sulfate in 10 mM sodium dihydrogenphosphate buffer (pH 7.5). This method resulted in a clear separation of the adducts from normal nucleotides and other impurities and offered the advantage that both diastereomers appear in one spot on the TLC sheet (Fig. 2, a and b).

**Autoradiography and Quantitation of the Adducts.** The adduct spots were visualized on 20 × 25-cm X-ray X-OMAT film from Kodak in an autoradiography cassette equipped with intensifying screens. Using these autoradiograms, the adduct spots on the TLC sheet were marked exactly with a pencil and cut out. The chromatograms were transferred into polyethylene vials, and 8 ml of water were added. The radioactivity was measured by Cerenkov counting in a liquid scintillation counter. The background radiation on the TLC sheets was measured with chromatogram pieces of the same size that did not contain adduct spots and was subtracted from the adduct count rate. The counting efficiency Z of the liquid scintillation counter was determined by calibration with standard solutions (Z = activity in cpm/activity in dpm). The activity of [γ-³²P]ATP was calculated on the basis of the radioactivity on the calibration day, according to the information of the supplier.

The RAL of the NP1 method was calculated by the following equation:

\[
\text{RAL} = \frac{\text{cpm (adducts)}}{A \times Z \times M}
\]

where A = specific activity of [γ-³²P]ATP (in dpm/pmol), Z = counting efficiency of the counter, and M = the amount of the DNA (in pmol).

The LE (%) was calculated according to the equation below:

\[
\text{LE} = \frac{\text{cpm (adducts)}}{A \times Z \times P \times 100}
\]

where P = amount of the adduct standard (in fmol).

**Detection Sensitivity.** Aliquots from 1–40 nmol of calf thymus DNA treated with crotonaldehyde (see above) were labeled as described after NP1 enrichment, and the labeled adducts were chromatographed by PEI-cellulose chromatography. The lowest radioactivity at which the adduct spots could be clearly detected after subtraction of the background was 260 cpm. This was achieved with an aliquot of 0.92 nmol. The absolute detectable amount of DNA adduct is 92 amol in a total amount of 0.92 nmol of DNA according to a calculated RAL of 10⁻⁷. This means that a total amount of 92 amol of adduct can be detected. When using 10 μg of DNA, the detection limit is 92 amol adduct/32.4 nmol DNA or about 3 amol adduct/10⁹ amol DNA (i.e., 3 adducts/10⁹ nucleotides).

**Recovery.** A recovery of 38 ± 4.3% was determined by adding 35 fmol of adduct standard to 10 μg of untreated DNA either from calf thymus or isolated from Fischer 344 rats (RAL of 109 × 10⁻⁸). Then the complete labeling procedure is carried out.

**Calculation of the TD₅₀.** The TD₅₀ (the dose at which 50% of the animals develop hepatocellular carcinoma) is calculated on the basis of the results of the study of Chung et al. (21). In this study, male Fischer 344 rats were given drinking water containing 0.6 mM crotonaldehyde (low dose group) and 6 mM crotonaldehyde (high dose group) for 113 weeks. According to Chung et al. (21), rats in the low dose group drank 20 ml water/day, i.e., 0.84 mg crotonaldehyde/rat/day on average, and rats in the high dose group drank 15 ml water/day, i.e., 6.3 mg crotonaldehyde/rat/day. The weights of the rats were about 450 g from the 50th week until the end of the study. According to the time/body weight curves presented in the article by Chung et al. (21), the average weight during the study is about 450 g. A low dose of about 1.8 mg crotonaldehyde/kg/day and a high dose of 14.0 mg crotonaldehyde/kg/day are calculated from these data. Two animals of the low dose group consisting of 27 rats developed hepatocellular carcinoma, but none of the high dose group animals did so. This means that the TD₅₀ is about 12 mg/kg/day.

**Intake of Crotonaldehyde by Humans via Different Routes.** The intake of crotonaldehyde by humans was estimated on the basis of the concentrations of crotonaldehyde in different foodstuffs as presented in the literature and as shown in Table 1. For references regarding the content of crotonaldehyde in the different foodstuffs, beverages, and tobacco smoke, see also Table 1.

**Cancer Risk Assessment.** The cancer risk for the different routes of intake was estimated on the basis of the TD₅₀ and the intake via each route (e.g., meat, drinks, smoking, and so forth) and is demonstrated in Table 1.
Carcinogenic effects of crotonaldehyde are considered to depend on the formation of exocyclic 1,2-propanodeoxy-guanosine adducts (15, 16, 21, 25, 26, 27, 29). This type of adduct was actually found to be promutagenic and to cause serious DNA lesions that can lead to initiation of tumor cells and tumor progression (18, 30–32). Furthermore, these adducts persist to a certain extent (Fig. 3). One week after the last of repeated gavages, 69% of the original adduct level measured 1 day after the last gavage was present, and after 2 weeks, 19% was still present. The persistence is important for considering the steady-state situation after permanent intake of low doses and for the experimental simulation of a steady-state condition by repeated gavages of low doses. However, the adducts are repaired to some extent because 2 weeks after the last of repeated gavages, only 19% of the initial amount of adducts as measured directly 1 day after the last gavage was left. However, the decrease in adducts can also be explained, at least in part, by cell turn over. Levels of these DNA adducts in human tissue can be considered as an appropriate measure not only to determine the actual intake of this compound as “target dose” but also as a biochemical strain parameter for the genotoxic steps in the multistage concept of carcinogenicity, i.e., initiation of cancer cells and tumor progression. Because application of carcinogenic compounds cannot be performed in humans for ethical reasons, investigations in animals and models to transfer the animal results into the human situation are necessary. Cancer risk assessment on the basis of DNA adducts can be best performed by adduct level tumor incidence relationships; however, such relationships are, to our knowledge, presently available for only a few compounds, e.g., tamoxifen (33).

Chung et al. (21) found an incidence of 2 hepatocellular carcinomas/27 animals in a long-term carcinogenicity study with Fischer 344 rats after application of a dose of 1.8 mg crotonaldehyde/kg body weight/day (low dose). However, no carcinomas were found after administration of the high dose of approximately 14 mg crotonaldehyde/kg body weight/day. A TD$_{50}$ of 12 mg crotonaldehyde/kg body weight/day is calculated on the basis of a cancer incidence of 7.4% (2 hepatocellular carcinomas/27 animals) at a dose of 1.8 mg crotonaldehyde/kg body weight/day according to the results of Chung. Using this TD$_{50}$, we calculated a tentative cancer risk for the intake of crotonaldehyde via different routes and estimated the DNA adduct levels to which these intakes would lead on the basis of our results (Table 1). Because of a lack of knowledge with regard to pharmacokinetic and metabolism data of crotonaldehyde in humans, it is anticipated in this first approach that absorption, distribution, and metabolism of crotonaldehyde from food and beverages in humans is similar to absorption and pharmacokinetic data of crotonaldehyde from drinking water in rats and that humans are similarly sensitive in developing hepatocellular carcinoma by crotonaldehyde exposure as rats. Moreover, a linear extrapolation from the low dose administered by Chung et al. (21) to the low doses of intake from food has to be carried out because the exact shape of the dose-response curve is not known. In principle, different shapes of a dose-cancer response curve are conceivable. Both linear or sigmoidal (S-shaped) curves are discussed frequently (34, 35). In the case of extrapolation from high doses (TD$_{50}$ of 12 mg crotonaldehyde/kg/day) to very low doses of 0.01–0.8 µg crotonaldehyde/kg/day as measured in food, the cancer risk values are considered to be similar at these very low doses independently of the shape of the curve. Only in cases in which the sigmoidal dose-response curves are extremely flat at low doses, in particular, in the case of a threshold, can higher differences be expected, or extrapolation is impossible. In general, there are no threshold limits in the case of genotoxic carcinogens (34). A linear extrapolation of human cancer risk to

![Fig. 3. Persistence of the crotonaldehyde DNA adducts in the days after the last of 20 gavages of 10 mg crotonaldehyde/kg body weight over 4 weeks (5 gavages/week).](image-url)
low dose might also be appropriate under certain conditions even if the dose-response curve in animals has a strong sigmoidal shape because nonlinearities are flattened out due to different susceptibility in subpopulations (35). According to this approach, food and some alcoholic beverages contribute to a dietary intake (e.g., utilization of disinfectants, air contamination) and differences in the food may account for this discrepancy. The standard food of Altromin (Lage, Germany) we used provides an optimum supply of antioxidative vitamins, selenium, and indigestible fibers that are important for (a) chemoprotective effects and (b) induction of possible endogenous formation of crotonaldehyde and other α,β-unsaturated carbonyl compounds in particular lipid peroxidation products. Such other α,β-unsaturated carbonyl compounds can compete with crotonaldehyde for glutathione, the most important factor in detoxication of α,β-unsaturated carbonyl compounds (37), and thus lead to glutathione depletion. We used the same inbred rat strain, Fischer 344, as Nath and Chung (27). We think that even if the rats are from different suppliers or stocks, it is unlikely that the same inbred strain of rats could have genetic differences that could influence the crotonaldehyde DNA adduct levels to this extent. On the other hand, we cannot completely exclude the possibility that the strain might be genetically unstable during inbreeding over a longer time period. However, to date, we could not find any indications in the literature that could explain the contradictory results by genetic alterations within this inbred rat strain. Another difference between our studies and those of Nath and Chung (27) is the amount of DNA used. Whereas we used 10 μg of DNA in our standard method, a 20-fold higher amount of DNA was used by Nath and Chung (27). However, during the development of our method, we occasionally also used higher amounts of DNA (up to 1000 μg of either calf thymus DNA or Fischer 344 rat DNA) and could not find any background adducts of this type. Therefore, the difference may depend on reasons other than methodology.

Two possible reasons may account for the discrepancy between the results of the carcinogenicity study of Chung et al. (21) and those of our DNA binding study. One is the additional formation of adducts that cannot be measured with our method. Hecht et al. (38) recently reported that they found indications that paraldol-DNA adducts, which cannot be measured with our method, are formed by crotonaldehyde. The other is high cytotoxicity, which may contribute indirectly to genotoxicity via reactive oxygen species and can lead to increased promotion. A high cytotoxicity was described by Chung et al. (21) in their cancer study, and the authors explained the surprising effect that no cancer incidence was observed with the high dose of 14 mg crotonaldehyde/kg body weight, which is close to the TD50 of 12 mg crotonaldehyde/kg body weight with high cytotoxicity (21). We observed that adduct formation is only slightly higher.
after gavage of repeated doses of 10 mg crotonaldehyde/kg as compared with repeated doses of 1 mg crotonaldehyde/kg (Table 2), and Chung et al. (21) found that cytotoxicity is strongly increased at the higher dose of 14 mg crotonaldehyde/kg body weight as compared with the lower dose of 1.8 mg crotonaldehyde/kg. Both effects may contribute to the unexpected outcome of the cancer study with the high dose. Nevertheless, the lack of tumorigenicity at the high dose in the cancer study of Chung et al. (21) cannot be explained satisfactorily by these effects alone and remains unclear.

The risk estimation on the basis of the cancer study of Chung et al. (21) and the estimated daily intake leads to a relatively high risk from common dietary exposure that is even higher than the total liver cancer rate observed in the past for industrialized Western countries. This overestimation can be explained by the lack of reliable data. The risk estimation had to be performed on a TDI calculated from only one dose (see the detailed discussion above). Nevertheless, this is a first approach, and the results indicate that crotonaldehyde can play an important role in human carcinogenesis, and crotonaldehyde may contribute to liver cancer in Western industrialized countries to a considerable degree. More knowledge on the relationship between exposure via different routes (e.g., inhalation and alimentary intake) and DNA adduct levels, particularly at small doses comparable to dietary intakes, and the relationships between DNA adduct levels and carcinogenic potential in different organs is necessary to work out more reliable cancer risk estimation.

Acknowledgments

We are indebted to Prof. Fung-Lung Chung (American Health Foundation, Valhalla, NY) for comments on the manuscript. We thank Christel Fabian and Elisabeth Weinert for excellent technical assistance and Karl-Heinz Link for skillful assistance in the animal experiments.

References

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