Metabolic activation of furfuryl alcohol: formation of 2-methylfuranyl DNA adducts in *Salmonella typhimurium* strains expressing human sulfortransferase 1A1 and in FVB/N mice

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Furfuryl alcohol, formed by acid- and heat-induced dehydration from pentoses, is found in many foodstuffs. It induced renal tubule neoplasia in male B6C3F1 mice and nasal neoplasms in male F344/N rats in a study of the National Toxicology Program (NTP). However, furfuryl alcohol was negative in the standard Ames test and in a battery of *in vivo* mutagenicity tests. Here, we show that furfuryl alcohol is mutagenic in *Salmonella typhimurium* TA100 engineered for expression of human sulfortransferase (SULT) 1A1. This finding suggests that furfuryl alcohol is converted by intracellular sulfo conjugation to 2-sulfo-oxymethylfuran, an electrophile reacting with DNA. We detected nucleoside adducts of 2-oxymethyl-2'-deoxyadenosine and 2-oxymethyl-2'-deoxyguanosine in porcine liver DNA incubated with freshly prepared 2-sulfo-oxymethylfuran. The main adducts, N2-(furan-2-yl)methyl)-2'-deoxyguanosine (N2-MFdG) and N6-(furan-2-yl)methyl)-2'-deoxyadenosine (N6-MFdA) were synthesized. Their structures were verified by NMR and mass spectrometry. Liquid chromatography–tandem mass spectrometry methods for the quantification of both adducts were devised. N2-MFdG and N6-MFdA were detected in DNA of furfuryl alcohol-exposed *S.typhimurium* TA100 expressing SULT1A1 and in DNA of liver, lung and kidney of FVB/N mice that had received ~390 mg furfuryl alcohol/kg body wt/day via the drinking water for 28 days. In summary, furfuryl alcohol is converted by sulfo conjugation to a mutagen. The detection of N2-MFdG and N6-MFdA in renal DNA of furfuryl alcohol-treated mice suggests that the neoplasms observed in this tissue in the study of the NTP may have been induced by 2-sulfo-oxymethylfuran.

Introduction

The furan derivative furfuryl alcohol originates from thermal dehydration of pentoses. It is present in heat-processed foods, such as cocoa, tea, coffee, dehydrated orange products, cooked meat and milk products (1), often together with hepatocarcinogenic furfural, which can be reduced in organisms to furfuryl alcohol (2). Furthermore, furfuryl alcohol is used as flavoring substance (3). The maximized amount of furfuryl alcohol is formed by sulfonation of other aromatic compounds, such as dimethyl furfural and 2-formylfuran, sulfur trioxide-pyridine, hydrogen chloride solution or sodium cyanoborohydride (1 M solution). Other reagents and solvents (analytical grade) were used similarly. The resulting 2-sulfo-oxymethylfuran can react with nucleophilic sites of the DNA (Figure 1). Thus, the reaction with 2-sulfo-oxymethylfuran (16,17), both of which undergo sulfonation of the benzyl alcohol to generate highly reactive furfural acid esters. The hypothesis that furfuryl alcohol is also activated metabolically by sulfortransferases (SULTs) of the corresponding adducts in DNA (SULT1A1) and thereby mediate the tumorigenic activity of furfuryl alcohol observed in B6C3F1 mice. However, Surh et al. (15) did not observe any mutagenic activity of chemically synthesized 2-sulfo-oxymethylfuran in *S.typhimurium*. We suspect that 2-sulfo-oxymethylfuran, a short-lived and charged molecule, may not be able to penetrate bacterial cell membranes. To circumvent this problem, we conducted mutagenicity tests of furfuryl alcohol using *S.typhimurium* strains expressing human SULT. To better understand the formation of 2-sulfo-oxymethylfuran and its genotoxic consequences in bacteria and FVB/N mice, techniques for the determination of 2-sulfofuranylnyl adducts based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) and multiple reaction monitoring (MRM) were developed.

Materials and methods

**Chemicals**

Shrimp alkaline phosphatase (from *Pandalus borealis*), micrococcal nuclease (from *Staphylococcus aureus*) and calf spleen phosphodiesterase were purchased from Sigma (Steinheim, Germany). High-performance liquid chromatography (HPLC)-grade methanol, 2-propanol, formic acid and acetic acid were from Carl Roth GmbH (Karlsruhe, Germany). Stable isotope-labeled [15N5]dA and [15N5]dG were from Campro Scientific (Berlin, Germany). Sodium cyanoborohydride (1 M solution in anhydrous tetrahydrofuran), furfuryl alcohol, formylfuran, sulfur trioxide-pyridine, hydrogen chloride solution (1.25 M in methanol) and all other reagents and solvents (analytical grade) were from Sigma.

Abbreviations: dA, 2'-deoxyadenosine; dC, 2'-deoxycytidine; dG, 2'-deoxyguanosine; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LOD, limit of detection; MRM, multiple reaction monitoring; N6-MFdA, N6-(furan-2-yl)methyl)-2'-deoxyadenosine; N6-MFdG, N6-(furan-2-yl)methyl)-2'-deoxyguanosine; NTP, National Toxicology Program; SULT, sulfortransferase; UPLC, ultra performance liquid chromatography.

The carcinogenicity of furfuryl alcohol in rats and mice was examined in a 2-year inhalation study of the National Toxicology Program (NTP) (7). Exposure of male B6C3F1 mice was associated with increased incidences of renal tubule neoplasms, whereas male F344/N rats developed neoplasms of the nose. These results were summarized as ‘some evidence of carcinogenic activity’ (7). In contrast to the carcinogenic potential, the compiled results of genotoxicity studies indicated that mutagenicity of furfuryl alcohol is at best weak. It was described as non-genotoxic based on the results of standard assays with *Salmonella typhimurium* strains TA98, TA100 and TA102 (8.9). No induction of sister chromatid exchanges and micronuclei was found in bone marrow cells of male B6C3F1 mice that were injected intraperitoneally with furfuryl alcohol (7). Chinese hamster ovary cells showed a concentration-related increase in chromosomal aberrations, the effect being enlarged in the presence of rat liver S9 mix (10). In contrast, the chromosomal aberrations test was negative in the absence of S9 and equivocal in the presence of S9 in the NTP study (7). Taken together, the evidence for furfuryl alcohol-related mutagenicity is scarce and the mechanism(s) underlying its carcinogenic effects are yet unknown. Based on these data, the European Food Safety Authority Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids concluded that furfuryl alcohol does not pose a genotoxic hazard to humans (11).

Most carcinogens require metabolic activation to electrophilically reactive intermediates. The major route of furfuryl alcohol metabolic transformation is oxidation to furfural, which is further oxidized to furoic acid and then conjugated with glycine to yield furoylglycine (12). Following administration of [14C]furfuryl alcohol to rats, 83–88% of the radioactivity was excreted in the urine as furoylglycine (73–80%), furoic acid (1–6%) and furanacrylic acid (3–8%) (12). None of these metabolites present structural alerts of mutagenic potential. However, minor and/or reactive metabolites might have been overlooked in this study. The structural analogy to the rodent carcinogen 5-hydroxymethylfurfural (13–15) and 1-hydroxyethylpyruvyl (16,17), both of which undergo sulfonation of the benzyl alcohol to generate highly reactive furfural acid esters led to the hypothesis that furfuryl alcohol may also be activated metabolically by sulfortransferases (SULTs). The resulting 2-sulfo-oxymethylfuran may react with nucleophilic sites of the DNA (Figure 1) and thereby mediate the tumorigenic activity of furfuryl alcohol observed in B6C3F1 mice. However, Surh et al. (15) did not observe any mutagenic activity of chemically synthesized 2-sulfo-oxymethylfuran in *S.typhimurium*. We suspect that 2-sulfo-oxymethylfuran, a short-lived and charged molecule, may not be able to penetrate bacterial cell membranes. To circumvent this problem, we conducted mutagenicity tests of furfuryl alcohol using *S.typhimurium* strains expressing human SULT. To better understand the formation of 2-sulfo-oxymethylfuran and its genotoxic consequences in bacteria and FVB/N mice, techniques for the determination of 2-sulfofuranylnyl adducts based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) and multiple reaction monitoring (MRM) were developed.
Synthesis of 2-sulfo-oxymethylfuran

Furfuryl alcohol (100 μl, 1.16 mmol) was mixed with 3 ml tetrahydrofuran and the solution was cooled to 0°C. After addition of 368 mg (2.32 mmol) sulfur trioxide–pyridine complex in 1 ml anhydrous N,N-dimethylformamide, the mixture was stirred for 5 h. Then, 6.9 ml of a 500 mM solution of sodium methoxide (3.45 mmol) was added. The resulting suspension was centrifuged and the supernatant was added dropwise to 80 ml of anhydrous diethyl ether. The precipitate containing the sodium salt of 2-sulfo-oxymethylfuran was filtered off, washed with ice-cold diethyl ether and stored under diethyl ether at −20°C. The identity of 2-sulfo-oxymethylfuran was confirmed by mass spectrometry. [EM](electrospray ionization (ESI)) m/z = 177 [M − H]−. It was used immediately for incubation with porcine liver DNA.

Synthesis of N-(2-(furan-2-yl)methyl)-2'-deoxyadenosine

A solution of 202 mg (0.75 mmol) 2'-deoxyadenosine (dA) and 62 μl (0.75 mmol) 2-formylfuran was prepared in 7.5 ml dry methanol containing 0.94 mM hydrogen chloride and stirred for 14 h at 60°C. After addition of 375 μl of a solution of sodium cyanoborohydride (1 M in tetrahydrofuran), the mixture was stirred for additional 14 h at 60°C. The solvent was evaporated and the residual was taken up in 3 ml water/methanol (1:1). The solution was filtered through a syringe filter (20 kDa) and the residual was taken up in 3 ml water/methanol (1:1). The solution was filtered through a syringe filter (20 kDa) and the supernatant was added dropwise to 80 ml of anhydrous diethyl ether. The precipitate was collected by centrifugation and was dried. The purity of the obtained product was >99% as determined by LC–UV–MS/MS. The isotope-labeled standards [13C10,15N5]-dA as starting material. The purity of [13C10,15N5]-dFdG and [13C15N4]-dMfdA was determined by LC–UV–MS/MS (>99%). MS: [13C10,15N5]-dFdG, m/z = 363.1 [M + H]+; [13C15N4]-dMfdA m/z = 337.1 [M + H]+.

**Reaction of porcine liver DNA with 2-sulfo-oxymethylfuran**

DNA (1 mg) was dissolved in 100 mM sodium phosphate buffer (pH 7) at a concentration of 1 mg/ml. Following addition of 1 mg (5 μmol) of the sodium salt of 2-sulfo-oxymethylfuran, the solution was incubated for 15 min at 37°C. The modified DNA was precipitated by addition of 100 μl of ice-cold 3 M sodium acetate (pH 5.2) and 700 μl of ice-cold 2-ethoxyethanol. Precipitation was completed for 45 min at −80°C. The suspension was centrifuged for 30 min at 15 000g and 4°C. The modified DNA was dried and re-dissolved in 0.5 ml water. The concentration of the DNA solution was determined from the absorbance at 260 nm using a nanodrop ND-1000 spectrophotometer (peqlab Biotechnologie, Erlangen, Germany). The DNA solution was stored at −80°C.

**Animal dosing with furfuryl alcohol**

Five male and five female FVB/N mice, purchased from Charles River Laboratories (Sulzfeld, Germany), were exposed to furfuryl alcohol in the drinking water containing 29.1 mM furfuryl alcohol for 28 days. The solution was stable for at least 2 weeks at room temperature as determined by LC–UV. Nevertheless, we provided fresh furfuryl alcohol solutions twice a week and weighed the drinking bottles to determine the consumption. During the experiment, animals consumed a daily average of 3.2 ml (female mice) and 3.4 ml (male mice) of the solution, corresponding to an uptake of 393 mg (female mice) and 391 mg (male mice) furfuryl alcohol/kg body wt. Control animals received water alone. After 28 days, all mice were killed by cervical dislocation. Liver, lungs, kidneys and colon mucosa were dissected and stored at −80°C. The DNA was isolated from homogenized tissues by phenol/chloroform extraction as described by Gupta (18), dissolved in a solution of 0.15 mM sodium citrate and 1.5 mM NaCl (pH 7.5) and stored at −80°C.

**Enzymatic digestion of DNA and solid-phase extraction of 2-methylfuranyl adducts**

A sample containing 100 μg DNA was dried together with fixed amounts of 1160 fmol [15N5]-dA and 143 fmol [13C10,15N5]-dFdG. The residue was taken up in 56 μl water and 16 μl of a solution containing 100 mM sodium succinate (pH 6.0) and 50 mM CaCl2 was added. A 24 μl aliquot of calf spleen phosphodiesterase (2.2 μl/mg DNA) and a pyrococcus nuclease (110 μl/mg) was added and the sample was incubated at 37°C for 4 h. After adding, 38 μl 0.5 M Tris (pH 10.9) and 3 ml shrimp alkaline phosphatase (1 U/ml), the incubation was resumed at 37°C for 14 h. Five-fold amounts of solutions and enzymes were used for samples of 500 μg DNA. The DNA digest was diluted by addition of 600 μl water and centrifuged at 15 000g for 15 min. The adducts were enriched by solid-phase extraction using an Oasis HLB column (3 cm3, 60 mg; Waters), preconditioned with 3 ml methanol and 3 ml water. The nucleoside mixture was loaded onto the column and washed with 3 ml of water/methanol (95:5). DNA adducts were eluted with 3 ml methanol. After evaporation of the solvents, the residuals were taken up in 30 μl water/methanol (25:75). Samples were centrifuged at 15 000g for 15 min and the supernatant was used for mass spectrometric analysis.

**LC-MS/MS of 2-methylfuranyl adducts**

The adduct mixtures previously enriched by solid-phase extraction were subjected to analytical reversed phase chromatography using an Acquity ultraperformance liquid chromatography (UPLC) System (Waters) with a UPLC HSS T3 column (1.8 μm, 2.1 × 100 mm; Waters). Samples of 8 μl were injected and eluted with water (solvent A) and acetonitrile (solvent B), applying a 10 min gradient starting from 100 to 80% solvent A at a flow rate of 0.35 ml/min. Both solvents were acidified with 0.25% acetic acid and 0.25% formic acid.

The UPLC was connected to a Quattro Premier XE tandem quadrupole mass spectrometer (Waters) with an electrospray interface operated in the positive-ion mode. The fragmentation of 2-MFDG into the aglycone cation [2-MFDG+(2-(furan-2-yl)methyl)-guanine - H]+ (348.1 → 232.1) and the cleavage of the 2-methylfuranyl cation (348.1 → 81.0), were monitored via MRM, together with the corresponding fragmentations of the internal standard [13C10,15N5]-dMfdA (363.1 → 242.1 and 363.1 → 81.0). In addition, we recorded the fragmentation of 2-MFDG into [N5-methylguanine - H]+ (348.1 → 164.0). The transition resulting from the neutral loss of 2'-deoxyribose from N5-MFDG was used as a qualifier for the measurement.
Quantifier signal, and the other transitions served as qualifier signals. Levels of \(N^2\)-MFdG were calculated by the peak areas of the traces 348.1 → 232.1 (analyte) and 363.1 → 242.1 (internal standard). In a similar fashion, the signal derived from the fragmentation of \(N^4\)-MFdA into the aglycone \([N^4-(furan-2-y1)methyl]-\text{adenine} - H^+\) (332.1 → 216.1) was used as a quantifier signal and the signal derived of the 2-methylfuranyl cation (321.2 → 81.0) was used as a qualifier signal. The amount of \(N^4\)-MFdA was calculated by the peak area of the ribose neutral loss from the internal standard \([^{15}\text{N}]N^4\)-MFdA (337.1 → 221.1).

The tune parameters for \(N^2\)-MFdG detection were as follows: temperature of the electrospray source: 110°C; desolvation temperature: 450°C; desolvation gas: nitrogen (850 l/h); cone gas: nitrogen (50 l/h); collision gas: argon (indicated cell pressure ~ 5.10 \(^{-3}\) mbar). For the fragmentation of \(N^2\)-MFdG, collision energies were 11, 25 and 35 eV for the transitions 348.1 → 232.1, 348.1 → 164.0 and 348.1 → 81.0, respectively. The dwell time was set to 100 ms and capillary voltage was set to 2.5 kV. The cone and RF1 lens voltages were 20 and 33 eV for the transitions 332.1 → 216.1 and 332.1 → 81.0, respectively.

Data acquisition and handling were performed with Mass Lynx software.

To determine the limit of detection (LOD), samples of blank porcine liver DNA were spiked with different amounts of \(N^2\)-MFdG and \(N^4\)-MFdA. The LODs (signal-to-noise ratio = 4) were 0.4 and 0.16 fmol for \(N^2\)-MFdG and \(N^4\)-MFdA, respectively. This corresponds to 0.1 molecules per 10⁶ nucleosides and 0.04 molecules per 10⁶ nucleosides for \(N^2\)-MFdG and \(N^4\)-MFdA, respectively, if 500 µg DNA is used for the analysis.

**Bacterial strains and mutagenicity experiments**

The *S. typhimurium* strain TA100 was kindly provided by B.N. Ames (Berkeley, CA). Strains TA100-SULT1A1 and TA100-SULT1A1/Y have been described elsewhere (19,20). Both strains express the reference sequence of S. typhimurium CA. Strains TA100-SULT1A1 and TA100-SULT1A1/Y have been described elsewhere (19,20). The bacterial suspension (100 µl) and the test compound (in 100 µl water) were added to a glass tube containing 400 µl of 100 mM MgSO₄. After incubation for 60 min at 37°C, 2.0 ml of 45°C warm soft agar (5.5 mg/ml agar, 5.5 mg/ml NaCl, 2.0 mg/ml neomycin (TA100-SULT1A1) and 2.0 mg/ml neomycin (TA100-SULT1A1/Y) or in the absence of antibiotics (TA100)). The plates were centrifuged, suspended in medium A (1.6 g/l Bacto Nutrient Broth, 5 g/l NaCl, 50 g/l M biotin, 50 µM histidine, 50 µM tryptophan, 25 mM sodium phosphate buffer, pH 7.4) and the mixture was poured onto a Petri dish containing 24 ml of minimal medium A (1.6 g/l Bacto Nutrient Broth, 5 g/l NaCl, 0.04 molecules per 10⁸ nucleosides for \(N^4\)-MFdA and \(N^2\)-MFdG, respectively.

**Results**

**Mutagenicity of furfuryl alcohol**

The substituted furan was not mutagenic in the *S. typhimurium* strain TA100 (Figure 2). To test the hypothesis that SULT activate furfuryl alcohol by conversion into the electrophilic sulfuric acid ester 2-sulfo-oxy methylfuran, we studied mutagenicity using two different TA100-derived strains expressing human SULT1A1 (19,20). Mutagenicity increased in a dose-dependent manner from 3 to 200 nmol furfuryl alcohol per plate, resulting in a maximum of ~400 revertant colonies per plate. The mutant frequencies calculated from the initial slope of the dose–response curves were four revertants per nanomoles and nine revertants per nanomoles for TA100-SULT1A1 and TA100-SULT1A1/Y, respectively.

**Formation of \(N^2\)-MFdG and \(N^4\)-MFdA in cell-free systems**

In order to detect putative 2-methylfuran adducts, porcine liver DNA was incubated with freshly synthesized 2-sulfo-oxy methylfuran, the proposed active metabolite of furfuryl alcohol (15). The DNA was digested and analyzed by an LC–MS/MS-screening approach to detect putative nucleoside adducts via the parent scan for the cleavage of the expected adduct molecule, the cation of 2-methylfuran (m/z = 81). Scanning for molecules that lost a fragment of m/z = 81 revealed pronounced signals for possible adducts of dA, dG and 2′-deoxyxycytidine (dC) with calculated mass-to-charge ratios of 332.1, 348.1 and 308.1, respectively (Figure 3). In addition to the cleavage of the 2-methylfuranyl moiety, the expected nucleoside adducts were predicted to undergo a neutral loss of the 2′-deoxyribosyl ring (x → y + 1). The LC–MS/MS MRM analysis of the DNA digest showed that four signals reflecting the fragmentation of the 2-methylfurfural unit from putative adducts of dA, dG and dC were accompanied by eluting peaks for the neutral loss of a deoxyribosyl fragment: The signals for the adducts of dA (8.28 min), dG (7.05 min) and dC (3.65 min and 3.85 min) (supplementary Figure S1 is available at Carcinogenesis Online; the capital S denotes that the figure can be found in the supplemental material). These transitions indicating the presence of 2-methylfuranyl nucleoside adducts were also detected when reaction mixtures containing individual nucleosides (dA, dG or dC) and 2-sulfo-oxy methylfuran were analyzed (data not shown), whereas the corresponding peaks were absent when a digest of untreated DNA was analyzed (supplementary Figure S2 is available at Carcinogenesis Online). The chromatogram for putative thymidine adducts extracted from the parent scan of the DNA digest was devoid of intense signals (Figure 3). Likewise, signals reflecting the neutral loss of the ribose moiety were not found in incubation mixtures containing either DNA or thymidine and 2-sulfo-oxy methylfuran.

In summary, the screening results were indicative for the formation of one 2-methylfuranyl adduct for each of the nucleosides dA and dG, whereas two 2-methylfuranyl adducts of dC may be formed. Earlier reports about DNA adducts from reactions with sulfuric acid esters showed that exocyclic nitrogens are predetermined for a nucleophilic attack at the electrophilic carbon atom, as observed for sulfate esters of tamoxifen (22), 1-methylpyrene (16) and estragole (23). Thus, the main nucleoside adducts observed in the presence of 2-sulfo-oxy methylfuran may originate from a reaction as depicted in Figure 1. We synthesized \(N^6\)-MFdA by reductive amination of the imine formed by dA and 2-formylfuran (24) and \(N^2\)-MFdG by nucleophilic substitution from dG and 2-sulfo-oxy methylfuran. LC–MS/MS analyses showed that the adduct standards \(N^6\)-MFdA and \(N^2\)-MFdG co-eluted with the respective adducts observed after incubation of DNA with 2-sulfo-oxy methylfuran (Figure 3). The MS/MS scan confirmed that fragmentation patterns of the standard substances were identical to those observed in the digest of porcine liver DNA incubated with 2-sulfo-oxy methylfuran. Three fragmentation reactions of \(N^2\)-MFdG produced signals with comparable peak areas, including the neutral loss of the deoxyribosyl unit.
NMR spectra corroborated that the structures of 2-methylfuran moiety linked to the exocyclic nitrogen, N\textsubscript{164.0}) (Figure 4). For the presence of 167/C\textsubscript{3} MRM of the DNA of TA100-SULT1A1

The 2-methylfuran adduct levels determined in the DNA of TA100 strains-expressing SULT1A1 are summarized in Table I. In the absence of furfuryl alcohol in the incubation medium, N\textsuperscript{2}-MFdG and N\textsuperscript{6}-MFdA were not detected in any of the bacterial DNA samples. Likewise, 2-methylfuran adducts were not formed in the parental strain TA100 exposed to furfuryl alcohol. N\textsuperscript{2}-MFdG and N\textsuperscript{6}-MFdA were detected in DNA of TA100 strains-expressing SULT1A1 treated with furfuryl alcohol. Levels of N\textsuperscript{6}-MFdA were usually close to the LOD, even after incubation with relatively high concentrations of furfuryl alcohol. In comparison, N\textsuperscript{2}-MFdG was well detectable.

N\textsuperscript{2}-MFdG and N\textsuperscript{6}-MFdA in furfuryl alcohol-exposed FVB/N mice

The occurrence of 2-methylfuran adducts in animals was initially tested by treating three FVB/N mice with a single intraperitoneal dose of 400 mg furfuryl alcohol/kg body wt. After 20 min, the mice were killed, hepatic DNA samples were isolated and analyzed for N\textsuperscript{2}-MFdG and N\textsuperscript{6}-MFdA. Using only 100 µg DNA, N\textsuperscript{6}-MFdA signals were below the LOD. In contrast, N\textsuperscript{2}-MFdG was clearly detectable with levels between 4 and 11.3 N\textsuperscript{2}-MFdG per 10\textsuperscript{8} nucleosides but absent in the liver of untreated mice (data not shown). This suggested that endogenous murine SULTs also activate furfuryl alcohol. In the following study, formation of N\textsuperscript{2}-MFdG and N\textsuperscript{6}-MFdA was monitored in male and female FVB/N mice consuming furfuryl alcohol in the drinking water for 28 days. The addition of furfuryl alcohol to the drinking water reduced the consumption by ~25–30% from 0.2 ml water/g body wt down to an average 0.14 ml aqueous solution/g body wt (male and female mice). The resulting daily dose of furfuryl alcohol was 391 mg/kg body wt for males and 393 mg/kg body wt for female mice. The uptake of standard chow was not influenced by furfuryl alcohol consumption. Likewise, development of body weight, behavior of the animals and macroscopic appearance of the investigated tissues (liver, kidney, lung and colon) of exposed mice were unobtrusive.

Levels of N\textsuperscript{2}-MFdG and N\textsuperscript{6}-MFdA were determined using 500 µg DNA for the analyses rather than 50 µg as in the initial experiment. In case of pulmonary DNA only, ~250 µg/mouse was available. Levels of colon mucosa yielded only ~50–100 µg DNA per mouse. Therefore, all colon mucosa samples of five animals in one group were pooled. The adduct levels were similar in liver, kidney and lung with 0.6–5 N\textsuperscript{2}-MFdG per 10\textsuperscript{8} nucleosides and 0.03–0.3 N\textsuperscript{6}-MFdA per 10\textsuperscript{8} nucleosides (Figure 6). In contrast, N\textsuperscript{2}-MFdG and N\textsuperscript{6}-MFdA were not detected in colon mucosa. In liver, kidney and lung, N\textsuperscript{6}-MFdG levels were generally 10- to 20-fold higher compared with those of N\textsuperscript{6}-MFdA. The latter were close, or in some samples even below, the LOD (0.04 N\textsuperscript{6}-MFdA per 10\textsuperscript{8} nucleosides). In contrast, the levels of N\textsuperscript{2}-MFdG were 8- to 50-fold higher than the LOD (0.1 N\textsuperscript{2}-MFdG per 10\textsuperscript{8} nucleosides), which strengthens the reliability of N\textsuperscript{2}-MFdG determination. Except for hepatic N\textsuperscript{2}-MFdG, comparison of the median adduct values indicate that levels of N\textsuperscript{2}-MFdG and N\textsuperscript{6}-MFdA were lower in male compared with female mice (Figure 6).

Discussion

5-Hydroxymethylfurfural, a structural analog of furfuryl alcohol, is converted to the sulfonic acid ester 5-sulfo-oxymethylfurfural in FVB/N mice (13). In contrast to the sulfonic acid ester of furfuryl alcohol, 5-sulfo-oxymethylfurfural was mutagenic in the standard

Fig. 3. 2-Methylfuranyl nucleoside adducts in porcine liver DNA incubated with 2-sulfo-oxymethylfurfural. Using the LC-MS/MS parent scan technique, all molecules with daughter ions of m/z = 81, the mass of the positively charged 2-methylfuranyl fragment, were detected. The extraction of parent mass chromatograms showed two to three signals for each of the mass-to-charge ratios 332.1, 348.1 and 308.1, which were indicative of 2-methylfuranyl adducts of dA (dAMF), dG (dGMF) and dC (dCMF), respectively. The peaks were absent when untreated DNA was analyzed (supplementary Figure S2 is available at Carcinogenesis Online). The gray peaks were accompanied by co-eluting signals reflecting the neutral loss of a deoxyribosyl unit, the cleavage of the 2-methylfuran cation (332.1 to 81.0) and the release of the neutral loss of the pentose moiety linked to the exocyclic nitrogen, N\textsubscript{2}-MFdG and transition 363.1 → 308.1, which were indicative of 2-MFdG and transition 348.1 → 232.1, which were indicative of 2-MFdG and transition 348.1 → 232.1. In contrast, N\textsuperscript{2}-MFdG was clearly detectable with levels between 4 and 11.3 N\textsuperscript{2}-MFdG per 10\textsuperscript{8} nucleosides but absent in the liver of untreated mice (data not shown). This suggested that endogenous murine SULTs also activate furfuryl alcohol. In the following study, formation of N\textsuperscript{2}-MFdG and N\textsuperscript{6}-MFdA was monitored in male and female FVB/N mice consuming furfuryl alcohol in the drinking water for 28 days. The addition of furfuryl alcohol to the drinking water reduced the consumption by ~25–30% from 0.2 ml water/g body wt down to an average 0.14 ml aqueous solution/g body wt (male and female mice). The resulting daily dose of furfuryl alcohol was 391 mg/kg body wt for males and 393 mg/kg body wt for female mice. The uptake of standard chow was not influenced by furfuryl alcohol consumption. Likewise, development of body weight, behavior of the animals and macroscopic appearance of the investigated tissues (liver, kidney, lung and colon) of exposed mice were unobtrusive.

Levels of N\textsuperscript{2}-MFdG and N\textsuperscript{6}-MFdA were determined using 500 µg DNA for the analyses rather than 50 µg as in the initial experiment. In case of pulmonary DNA only, ~250 µg/mouse was available. Samples of colon mucosa yielded only ~50–100 µg DNA per mouse. Therefore, all colon mucosa samples of five animals in one group were pooled. The adduct levels were similar in liver, kidney and lung with 0.8–5 N\textsuperscript{2}-MFdG per 10\textsuperscript{8} nucleosides and 0.03–0.3 N\textsuperscript{6}-MFdA per 10\textsuperscript{8} nucleosides (Figure 6). In contrast, N\textsuperscript{2}-MFdG and N\textsuperscript{6}-MFdA were not detected in colon mucosa. In liver, kidney and lung, N\textsuperscript{6}-MFdG levels were generally 10- to 20-fold higher compared with those of N\textsuperscript{6}-MFdA. The latter were close, or in some samples even below, the LOD (0.04 N\textsuperscript{6}-MFdA per 10\textsuperscript{8} nucleosides). In contrast, the levels of N\textsuperscript{2}-MFdG were 8- to 50-fold higher than the LOD (0.1 N\textsuperscript{2}-MFdG per 10\textsuperscript{8} nucleosides), which strengthens the reliability of N\textsuperscript{2}-MFdG determination. Except for hepatic N\textsuperscript{2}-MFdG, comparison of the median adduct values indicate that levels of N\textsuperscript{2}-MFdG and N\textsuperscript{6}-MFdA were lower in male compared with female mice (Figure 6).

Discussion

5-Hydroxymethylfurfural, a structural analog of furfuryl alcohol, is converted to the sulfonic acid ester 5-sulfo-oxymethylfurfural in FVB/N mice (13). In contrast to the sulfonic acid ester of furfuryl alcohol, 5-sulfo-oxymethylfurfural was mutagenic in the standard
Ames test (15). This discrepancy may be explained by the greatly differing half-life times of the sulfuric acid esters in aqueous solutions. 5-Sulfo-oxymethylfurfural is rather stable (t_{1/2} = 120 min), whereas 2-sulfo-oxymethylfuran lacking the stabilizing aldehyde function is short-lived (t_{1/2} = 20 s) (25). As a consequence, 2-sulfo-oxymethylfuran may be hydrolized before effectively penetrating the bacterial cell membranes, which explains the lack of mutagenicity in the Ames test (15). Here, we showed that furfuryl alcohol is mutagenic in \textit{S. typhimurium} strains TA100 engineered for the expression of human SULT1A1. The SULT was selected because of its dominant role in the activation of other benzylic alcohols, its broad substrate tolerance and its high expression levels in numerous tissues (26,27). The plausible explanation for the mutagenic effect of furfuryl alcohol in TA100-SULT1A1 is that 2-sulfo-oxymethylfuran is generated intracellularly in proximity to the bacterial DNA leading to the formation of 2-methylfuranyl adducts. The instability of 2-sulfo-oxymethylfuran prohibited the direct analysis of the sulfo conjugation of furfuryl alcohol. However, we detected specific adducts N²-MFdG and N⁶-MFdA in the DNA of furfuryl alcohol-treated TA100-SULT1A1. These were previously identified as the predominant adducts in porcine liver DNA incubated with 2-sulfo-oxymethylfuran \textit{in vitro}. The adduct levels observed in the DNA of SULT-expressing bacteria correlated with the furfuryl alcohol concentration in the incubation medium and the mutagenic activity (Table 1). These findings strongly support the concept that furfuryl alcohol was activated via SULT-catalyzed sulfo conjugation and that formation of covalent 2-methylfuranyl adducts caused the mutagenic effect observed in SULT-expressing TA100 strains.

In order to study whether endogenous murine SULT also activate furfuryl alcohol, mice received furfuryl alcohol for 28 days in the drinking water. It is probable that a steady-state level of DNA adducts was reached in this time, with adduct formation and adduct removal being balanced (28). DNA samples of liver, kidney and lung contained 2-methylfuranyl adducts in comparable levels (Figure 6), indicating that murine SULTs can activate furfuryl alcohol and that the reaction also occurs \textit{in vivo}.

The formation of methylfuranyl adducts in mice depends on furfuryl alcohol sulfo conjugation, directed transport of the sulfate esters and DNA repair, all of which are organ-specific processes. The extent of sulfo conjugation in a particular organ is not only determined by the SULT expression but also by the cellular concentration of the cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) (29). It is of note that data on SULT expression in murine organs on the protein level is lacking as well as data on furfuryl alcohol sulfo conjugation capacity of single murine SULT forms. Directed transport processes may greatly alter the concentration of sulfate esters in individual organs (30–32). Taken together, the levels of DNA adducts in specific organs cannot be simply correlated to SULT expression. Murine SULT1A1 and SULT1D1 are probably involved in furfuryl alcohol sulfo conjugation. In a recent paper, we reported that furfuryl alcohol exhibited mutagenic activity in \textit{S. typhimurium} TA100 strains expressing murine SULT1A1 and SULT1D1 (33). The mRNA level of these SULT forms reported in a comprehensive study from Alnouti \textit{et al.} (34) indicate that liver and lung of mice contain relatively high amounts of murine SULT1A1, whereas SULT1D1 is strongly expressed in kidney. Thus, the detection of equivalent methylfuran levels in the organs of furfuryl alcohol-treated mice is not surprising. More information is needed about furfuryl sulfo conjugation by other murine SULT and about directed transport of 2-sulfo-oxymethylfuran in order to discuss methylfuran adduct distribution.

A tissue-related association between levels of 2-methylfuranyl adducts and rodent tumor incidence induced by lifetime inhalative exposure to furfuryl alcohol as described in the report NTP TR 482 was not observed (7). Male B6C3F1 mice in the high-dose group of the inhalational study received ~60 mg furfuryl alcohol/kg body wt/day over a period of 2 years and developed renal tubule neoplasms, which may originate from 2-methylfuranyl adducts in the kidney. In contrast, neither neoplastic effects nor 2-methylfuranyl adducts were detectable in the colon of furfuryl alcohol-treated mice. Finding of \textit{N²}-MFdG and \textit{N⁶}-MFdA in other tissues did not correlate with the induction of tumors at specific sites. This is not surprising due to several reasons. On the one hand, the tumorigenic efficiency of a particular DNA adduct level may vary between tissues and sexes (28,35). On the other hand, the correlation of results is hindered by important technical differences between the studies: Firstly, distribution and metabolism of furfuryl alcohol may be greatly affected by the uptake route, which were epithelia of respiratory tract and lung in the inhalation study and the gastrointestinal tract in our experiment. Secondly, animals in the high-dose group of the NTP study received only ~60 mg furfuryl alcohol/kg body wt/day (7). As a consequence, certain neoplastic effects may have been below the LOD. This is supported by an interesting study of Ottender \textit{et al.} (35). The authors correlated hepatic DNA adduct levels and the incidences of liver tumors induced by 10 common carcinogens in rodents in order to test the potential use of DNA adduct levels as cancer risk markers. Hepatic steady-state

![Fig. 4. Fragmentation pattern of N²-MFdG (A) and N⁶-MFdA (B) observed by positive ESI MS/MS collision-induced dissociation. Principal fragmentation ions of N²-MFdG were m/z = 232.1 (the aglycone of N²-MFdG), m/z = 81.0 (the cation of 2-methylfuran), m/z = 164.0 (protonated N²-methylguanine) and m/z = 117.0 (protonated 2'-deoxyribose). The fragmentation pattern of N⁶-MFdA (B) was comparable.](https://academic.oup.com/carcin/article-abstract/32/10/1533/2463379)
Fig. 5. LC–MS/MS chromatograms of a digested DNA sample from furfuryl alcohol-treated TA100-SULT1A1, which were exposed to 167 μM furfuryl alcohol for 1 h (corresponds to 100 nmol furfuryl alcohol per plate in the mutagenicity assay). The chromatograms for N²-MFdG were derived from the fragmentations 348.1 → 232.1 (first panel) and 348.1 → 81.0 (second panel), which were monitored together with the transitions 363.1 → 242.1 (third panel) and 363.1 → 81.0 (fourth panel) of the internal isotope-labeled standard [13C10,15N5]N²-MFdG (310 fmol/injection). The ratio of peak areas for the transition 348.1 → 232.1 ([13C10,15N5]N²-MFdG) and for the transition 363.1 → 242.1 ([13C10,15N5]N²-MFdG) was used to calculate the N²-MFdG content of the DNA. Corresponding chromatograms of N²-MFdA analyses and of DNA from unexposed TA100-SULT1A1 bacteria are shown in supplementary Figures S5 and S6, available at Carcinogenesis Online, respectively.

Fig. 6. Levels of N²-MFdG (A) and N⁶-MFdA (B) detected by LC–MS/MS MRM in liver, kidney and lung of FVB/N mice exposed to furfuryl alcohol in the drinking water for 28 days. Animals were killed and DNA adduct levels were determined. The circles denote adduct levels in single animals; gray fillings mark measurements with signal-to-noise ratios < 4. DNA samples of control animals were devoid of 2-methylfuran adducts. The bars designate median values. Wilcoxon rank sum test P < 0.05.

Table 1. N²-MFdG and N⁶-MFdA in bacterial DNA as determined by LC–MS/MS MRM

<table>
<thead>
<tr>
<th>Strain</th>
<th>Furfuryl alcohol, nmol/plate</th>
<th>Revertants N⁶-MFdA per 10⁸ nucleosides</th>
<th>N²-MFdG per 10⁸ nucleosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA100</td>
<td>0</td>
<td>118 ± 9 n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>130 ± 8 n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>TA100-SULT1A11'1</td>
<td>0</td>
<td>86 ± 3 n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>176 ± 23 n.d.</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>361 ± 26 n.d.</td>
<td>2.60</td>
</tr>
<tr>
<td>TA100-SULT1A11'1Y</td>
<td>0</td>
<td>56 ± 5 n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>208 ± 19 n.d.</td>
<td>1.29</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>404 ± 47 n.d.</td>
<td>5.67</td>
</tr>
</tbody>
</table>

n.d., not detectable (LODs for the analysis of 500 μg DNA: 0.04 N⁶-MFdA per 10⁸ nucleosides; 0.1 N²-MFdG per 10⁸ nucleosides); mutagenicity results are mean values and SE of three plates.

adduct levels measured at a given dose after a minimum exposure of 10 days were normalized to the dose which resulted in a 50% incidence of hepatic tumors (TD50) under the conditions of 2 years carcinogenicity assay. Calculated adduct levels at the TD50 ranged from 53 adducts per 10⁸ nucleosides for aflatoxin B1 in rats to ~5500 adducts per 10⁸ nucleosides observed for 2-acetylaminoﬂuorenone in mice. Hepatic levels of N²-MFdG and N⁶-MFdA in FVB/N mice in our study were low (taken together ~1.8 adducts per 10⁸ nucleosides), suggesting that the hepatocarcinogenic risk of ~390 mg furfuryl alcohol/kg body wt/day is limited. Accordingly, hepatic tumors were absent in the NTP study, in which rodents received only ~60 mg furfuryl alcohol/kg body wt/day.

Compared with most of the carcinogens mentioned in the study of Otteneder and Lutz, such as aflatoxin B1, 2-acetylaminoﬂuorenone, 4-aminobiphenyl and dimethylnitrosamine, doses of furfuryl alcohol required for detection of N²-MFdG and N⁶-MFdA in murine DNA or for the induction of renal tumors in mice were high. However, human intake of furfuryl alcohol also exceeds those of other carcinogens. As a flavoring food additive, the possible average daily intake has been estimated to amount to 130 μg/kg body wt (4), which is 3100-fold less than the dosage used in the study presented here. The total intake of furfuryl alcohol with food is considerably higher. Concentrations in roasted coffee beans were in the range of 1.5–3 mg/g furfuryl alcohol, resulting in intake of between 15 and 30 mg furfuryl alcohol per serving coffee (M.Murkovic, manuscript in preparation). Our findings show that furfuryl alcohol is converted into a genotoxic agent by sulfo conjugation. Thus, the recent conclusion of the European Food Safety Authority that human furfuryl alcohol intake is not a concern has to be challenged (11). Reconsideration of the carcinogenic potential requires quantitative data about the furfuryl alcohol content in daily nutrition and experimental results about sulfo conjugation of furfuryl alcohol catalyzed by murine or human SULT in order to better interpret results from animal experiments. In addition, there is a lack of information about the extent of sulfo conjugation compared with competing oxidative pathways of furfuryl alcohol in humans and mice.

In summary, furfuryl alcohol was shown to be mutagenic in TA100-SULT1A11'1 but not in the parental strain. In the DNA of TA100-SULT1A11'1 treated with furfuryl alcohol, the nucleoside adducts N²-MFdG and N⁶-MFdA were detected which were also present in porcine liver DNA incubated with chemically prepared 2-sulfo-oxy methylfuran. These data demonstrate that SULT1A1
bioactivates furfuryl alcohol and that 2-sulfo-oxyethylfuran is the metabolite that forms DNA adducts and induces mutations in this model. Moreover, the same activation occurs in murine tissues in vivo, including a target tissue of carcinogenesis, the kidney of male mice. Therefore, we postulate that the observed DNA adducts are causally involved in the carcinogenesis induced by furfuryl alcohol.

Supplementary material
Supplementary Figures S1–S6 can be found at http://carcin.oxfordjournals.org/

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References

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