

Toxicity studies with 5-hydroxymethylfurfural and its metabolite 5-sulphooxymethylfurfural in wild-type mice and transgenic mice expressing human sulphotransferases 1A1 and 1A2

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Abstract 5-Sulphooxymethylfurfural (SMF), an electrophilic metabolite of the abundant Maillard product 5-hydroxymethylfurfural (HMF), was intraperitoneally administered to FVB/N mice. At a dosage of 250 mg/kg, most animals died after 5–11 days due to massive damage to proximal tubules. At lower dosages, administered repeatedly, tubules also were the major target of toxicity, with regeneration and atypical hyperplasia occurring at later periods. Additionally, hepatotoxic effects and serositis of peritoneal tissues were observed. SMF is a minor metabolite of HMF in conventional mice, but HMF is an excellent substrate for a major sulphotransferase (hSULT1A1) in humans. Parental FVB/N mice and FVB/N-hSULT1A1/2 mice, carrying multiple copies of the hSULT1A1/2 gene cluster, were exposed to HMF in drinking water (0, 134 and 536 mg/kg body mass/day) for 12 weeks. Nephrotoxic effects and enhanced proliferation of hepatocytes were only detected at the high dosage. They were mild and, surprisingly, unaffected by hSULT1A1/2 expression. Thus, SMF was a potent nephrotoxicant when administered as a bolus, but did not reach levels sufficient to produce serious toxicity

when generated from HMF administered continuously via drinking water. This was even the case in transgenic mice expressing clearly higher HMF sulphation activity in liver and kidney than humans.

Keywords Nephrotoxicity · Hepatotoxicity · 5-Hydroxymethylfurfural · 5-Sulphooxymethylfurfural · Human sulphotransferases 1A1 and 1A2

Introduction

During the preparation of food numerous, new compounds are formed. One of them, present at high levels in many foods, is 5-hydroxymethylfurfural (HMF) (structural formula in Fig. 1). It is generated when reducing hexose moieties are heated in the presence of amino acids or proteins (Mauron 1981) and via acid-catalysed thermal dehydration of fructose, sucrose and glucose (Antal et al. 1990). Various studies conducted in the 1960s and 1970s consistently showed low acute and subchronic toxicity of HMF in mice and rats [reviewed by Ulbricht et al. (1984)]. However, HMF has a number of structural alerts (furan ring, α,β -unsaturated carbonyl group and allylic hydroxyl group) that point to a possible risk of genotoxic and carcinogenic activity. Indeed, it was reported that HMF initiates and promotes preneoplastic lesions (aberrant crypt foci) in rat colon (Zhang et al. 1993). HMF also demonstrated tumour-initiating activity in mouse skin, using 12-*O*-tetradecanoylphorbol-13-acetate as the promoter, the effect being statistically significant in the study conducted by Surh et al. (1994), but not in the initial study reported by Miyakawa et al. (1991). In a 2-year gavage study, HMF did not show evidence of carcinogenic activity in Fischer 344N rats of both sexes and in male B6C3F₁ mice (NTP 2010).

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However, in female B6C3F₁ mice, it clearly increased the incidence of hepatomas at dosages of 188 and 375 mg/kg body mass (5 times per week, equivalent to mean daily dosages of 134 and 268 mg/kg, respectively). At these doses, the incidence of hepatomas was 53 and 52%, respectively, compared to a value of 28% for the actual negative control and 22% for the mean historical negative control. The third dosage group (750 mg/kg body mass) was not included in the evaluation of carcinogenic potential because of the reduced survival and the presence of the treatment-related clinical signs. Older estimates of the mean daily intake of HMF from food are in the range of 30–150 mg per person, equivalent to 0.4–2.1 mg/kg body mass for a person of 70 kg (Ulbricht et al. 1984; Janzowski et al. 2000). Estimates from newer studies from Spain (Rufian-Henares and de la Cueva 2008), Norway (Husøy et al. 2008) and Germany (Abraham et al. 2011) are somewhat lower. A margin of exposure cannot be calculated, as appropriate carcinogenicity data are only available for two dosage levels. However, since HMF induced hepatocellular adenomas in 25% of all animals (female mice, after subtracting the spontaneous tumour incidence) even at the lowest dosage tested, it is clear the margin of exposure is below 60–300, which is exceptionally low in comparison with other food-borne carcinogens.

Surh and Tannenbaum (1994) found that HMF can be activated to an electrophilic sulphuric acid ester, SMF (Fig. 1), in the presence of rat hepatic cytosolic preparations fortified with the cofactor 3'-phosphoadenosine-5'-phosphosulphate. Topically applied to mouse skin, SMF exhibited higher skin tumour-initiating activity than the parent compound HMF (Surh et al. 1994). We demonstrated that biotransformation of HMF to SMF also occurs in the mouse in vivo (Monien et al. 2009). Nearly 500 ppm of the initial HMF dose reached the circulation as SMF; an unknown additional amount of SMF may have been further metabolized or reacted with cellular structures directly at the site of its formation. Interestingly, the most abundant human sulphotransferase (SULT), SULT1A1, showed higher catalytic efficiency toward HMF than any rodent form studied (Glatt and Sommer 2006). For this reason, mice and rats may not be ideal for testing the safety of HMF. Therefore, we used two alternative models. In the first model, we directly administered the reactive metabolite, SMF, to animals by

intraperitoneal injection. We choose this route of administration, as natural exposure to SMF occurs via transformation of HMF within the body, rather than direct intake in the diet. Moreover, it was unlikely that the oral administration of a reactive phase-2 metabolite would lead to an appreciable systemic bioavailability. The second model involved the application of HMF in drinking water to wild-type and transgenic mice expressing high levels of human SULT1A1 and SULT1A2 in many tissues, including colonic mucosa (Dobbernack et al. 2011). Both enzymes show high catalytic efficiency using HMF as the substrate (Glatt and Sommer 2006). In this model, SMF is generated within cells and, thus, its relative levels in different tissues and cells may differ from those occurring after intraperitoneal administration of SMF. Moreover, since the tissue distribution of human SULT1A1 and SULT1A2 is similar in humans and in the transgenic mice used (Dobbernack et al. 2011), this model may best reflect human exposure to SMF.

Materials and methods

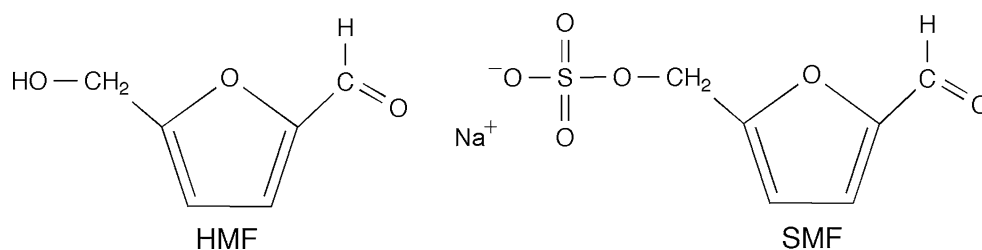
Test compounds

HMF (CAS 67-47-0, purity 99 ± 1%, determined by various methods) was a gift of Südzucker AG (Mannheim, Germany). SMF (CAS 159091-35-7; purity >99%, determined by various methods) was synthesized (Monien et al. 2009) in the laboratory of Dr. A. Seidel (Grosshansdorf, Germany).

Animals

FVB/N mice are particularly suited for gene-transfer into germ cells. Therefore, this strain was used. Wild-type FVB/N mice were purchased from Harlan-Winkelmann (Borchen, Germany) and acclimatized for a period of at least 7 days before treatment. Transgenic FVB/N mice, harbouring multiple copies of the human SULT1A1-SULT1A2 gene cluster in chromosome 9, were propagated as a homozygous line in our laboratory (Dobbernack et al. 2011). They were bred with wild-type FVB/N mice to produce hemizygous mice for the experiments (subsequently termed FVB/N-hSULT1A1/2 mice).

Fig. 1 Structural formulas of the compounds studied, 5-hydroxymethylfurfural (HMF) and 5-sulphoxymethylfurfural (SMF). SMF, a reactive metabolite of HMF, was synthesized and administered to mice as its sodium salt



Animals were kept under standard temperature, humidity and timed lighting conditions. They were fed a standard mouse chow, V1124-000 (Ssniff Spezialdiäten GmbH, Soest, Germany) and had free access to tap water.

Expression of endogenous SULTs in murine tissues undergoes pronounced ontogenetic changes in the first weeks after birth (Alnouti and Klaassen 2006). Treatment was started at an age of 8–12 weeks, when the animals had surpassed this phase. Due to high sexual dimorphisms of expression of endogenous SULTs (Alnouti and Klaassen 2006), both sexes were used when testing HMF. In contrast, the ultimate genotoxicant SMF was only investigated in male wild-type mice. In the experiments, animals were housed individually. All animal experiments were performed with permission of the Landesamt für Umwelt, Gesundheit und Verbraucherschutz of the State of Brandenburg.

HMF sulphation rates in hepatic cytosolic fractions (expressed as nmol/min/mg protein) were determined as 25 (wild-type males), 117 (wild-type females), 304 (hemizygous transgenic males), 374 (hemizygous transgenic females) and 67 (humans) nmol/min/mg protein (substrate concentration 2.5 mM; means of 2–4 animals or humans); the corresponding values for kidney were 22, 30, 214, 126 and 6 nmol/min/mg, respectively [for details see (Florian et al. 2012)]. The transgenic animals expressed hSULT1A1 and hSULT1A2 protein in all these tissues (Dobbernack et al. 2011). However, since hSULT1A1 levels were higher than those of hSULT1A2—like in humans—(Dobbernack et al. 2011) and HMF is a better substrate for the former enzyme (Glatt and Sommer 2006), the enhanced HMF sulphation activity in transgenic animals appears to be primarily mediated by hSULT1A1.

Treatment regimen

Background information

The experiments were primarily designed to study whether SMF, administered directly or formed metabolically from HMF, is able to induce the formation of aberrant crypt foci in the colon of mice. The results have been presented elsewhere (Florian et al. 2012). The same animals were used in the present study for investigating adverse effects in tissues other than colon.

The doses of HMF used [536 mg (4.3 mmol) and 134 mg (1.1 mmol)/kg body mass/d] were based on doses employed in the study of the National Toxicology Programme of the USA (NTP) (2010). In the first experiment, SMF was used at a dose [250 mg (1.1 mmol)/kg body mass/treatment] equimolar to the lower HMF dose. In a pilot experiment, we had administered this dose to a small number of animals to study the formation of DNA adducts

(the result was negative, but the ^{32}P post-labelling method used was inadequate for the detection of these adducts, as noticed later as). The animals appeared to tolerate this dose well, but were killed within 48 h. Therefore, we did not recognize delayed effects, a mistake manifested in experiment 1 with SMF (next paragraph).

Experiments with SMF

SMF has a half-life time of 2 h in aqueous solution at 37°C (Glatt and Sommer 2006). It was dissolved in saline immediately before use. It was verified that less than 2% of the test compound was decomposed before injection. In all experiments, it was administered intraperitoneally to young adult male FVB/N mice (8- to 12-week old at first treatment). In the *first experiment*, 35 animals received a single dose of 250 mg (1.1 mmol) SMF/kg. An equal number of animals was only treated with saline and served as control. Due to the dramatic effect of SMF in this experiment, not all control animals were required for histological analysis. The remaining 14 animals were used in a *second experiment* with the aim to better estimate the maximum tolerated dose level. Four animals per group were treated twice (with an interval of 7 days) at dosages of 31.25 mg (0.14 mmol), 62.5 mg (0.27 mmol) and 125 mg (0.55 mmol) SMF/kg. The other two animals were used as negative controls. The animals were killed 8 weeks after the last treatment. The *third (final) experiment* involved 105 animals divided into four treatment groups. The groups received four intraperitoneal administrations of the following test compounds at one-week intervals: 62.5 mg (0.27 mmol) SMF/kg (number of animals, $n = 35$), 125 mg (0.55 mmol) SMF/kg ($n = 15$), 10 mg azoxymethane/kg ($n = 20$) or the vehicle only (saline, $n = 35$), with intervals of 1 week between the treatments. The azoxymethane-treated animals were only used as a positive control for the induction of aberrant crypt foci and tumours in colon; other tissues were not histologically examined. Subgroups of animals were killed at varying times after the last treatment, as indicated in Table 3. Animals were randomly assigned to these groups and subgroups at the beginning of the experiment.

Experiment with HMF

HMF was given in the drinking water. The dosages were based on those used in a 2-year gavage study (750, 375 and 188 mg/kg body mass, 5 times per week) conducted in the NTP study (2010). The high and low dosages of that study are equivalent to 536 mg (4.3 mmol) and 134 mg (1.1 mmol) per kg body mass, respectively, if given daily rather than only five times a week. Male and female wild-type FVB/N and heterozygous FVB/N-hSULT1A1/2 animals (6 animals per group, 8 weeks old at the beginning of the treatment)

received these dosages in drinking water daily for 12 weeks. The concentration of HMF in the flasks was adjusted to the average daily water consumption. The drinking water was changed weekly. The stability of HMF in water over this period was verified using HPLC–UV analyses.

Tissue preparation and histology

The animals were killed by cervical dislocation. Organs were removed and prepared in a standardized procedure. A thorough pathologic anatomical examination was performed on each animal. Samples of various tissues (specified in the results section) were fixed in 4% buffered (pH 7.0) formalin for 24 h, embedded in paraffin, cut into 2- μ m sections and stained. Three different stainings were used for neighbouring sections: haematoxylin-eosin, Perl's blue and Okajima's stain for haemoglobin (Romeis 1989). Tissue sections were viewed under a light microscope (E 1000, Nikon, Düsseldorf, Germany).

In the study on HMF, renal damage was scored in coded samples from the individual animals based on the following five pathomorphological parameters: cell death, mononuclear infiltrates with regenerates, interstitial nephritis, tubulitis and tubular damage; each parameter got a score from 0 (no damage) to 3 (strong damage). They were added to give the total score, which therefore could range between 0 and 15. Scoring was performed by a veterinarian who had conducted her habilitation work on kidney pathology.

Immunohistochemical detection of proliferation marker

Ki-67-immunohistochemistry was performed in kidney and liver sections in HMF-treated and control animals in order to detect proliferative cells as described previously (Bauer-Marinovic et al. 2006). In short, slides were immersed in boiling Target Retrieval Solution (ChemMateTM, DakoCytomation, Hamburg, Germany) and incubated overnight with primary antibody for Ki-67 (TEC-3, DakoCytomation) diluted in antibody diluent (DakoCytomation). A polymer-conjugated secondary antibody [N-Histofine® Simple Stain Mouse MAX PO (Rat), Nichirei Bioscience, Tokyo, Japan] was applied followed by visualization via diaminobenzidine (DakoCytomation).

Results

Effects of SMF directly administered to mice

Experiment 1 (single treatment with 250 mg SMF/kg)

SMF-treated animals appeared apathetic compared to vehicle-treated controls in the first hours after the injection.

They recovered quickly and no obvious differences from the controls were noticed in the next 4 days. However, on days 5 and 6, several SMF-treated animals died and the others had developed impaired body conditions, such as rough fur, boarded abdomen, reluctance to move and hunched posture as well as lethargy or recumbency. A total of 20 of 35 animals died between days 5 and 11 after SMF treatment. Ten moribund animals were euthanized between days 6 and 9. The remaining five animals of the SMF group survived and were killed at varying later time points (2–40 weeks after the treatment) for preliminary histological analysis (which agreed with findings from the much more comprehensive final experiment and thus are not presented).

The mice killed in moribund state had pale and swollen kidneys as well as hepatomegaly on dissection. There was dark discoloration of the faeces. Acute necrosis of fatty tissue at the caudal pole of the right kidney was detected in one animal. Two animals showed adhesions between liver and intestine as well as between liver and kidney.

Histopathological lesions in SMF-treated animals, compared to controls, were observed in liver and kidneys (summarized in Table 1), but not in the other tissues investigated (myocardium, urinary bladder, lung, pancreas, spleen, stomach, duodenum, jejunum, ileum, caecum and

Table 1 Histopathological findings in moribund FVB/N mice killed 6–9 days after a single intraperitoneal treatment with SMF (250 mg/kg) compared to vehicle (saline)-treated mice

Tissue, lesion	Number of animals affected	
	SMF-treated (10 mice)	Controls (5 mice)
Kidney		
Tubular necrosis ^a		
none	0	5
in 50–75% of all tubules	4	0
in >75% of all tubules	6	0
Liver		
Necrosis		
none	0	5
focal, <10% of all hepatocytes	3	0
moderate, 10–30% of all hepatocytes	7	0
Nodular hyperplasia	3	0
Serositis	5	0
Coagulation necrosis	3	0
Pleomorphism of hepatocytes	4	0

Ten SMF-treated and five control animals were used. All evaluations were conducted on coded slides

^a Analysis was conducted with a light microscope with 100 \times magnification. For each animal, ten fields within the kidney cortex were randomly chosen for determining the per cent of damaged tubules

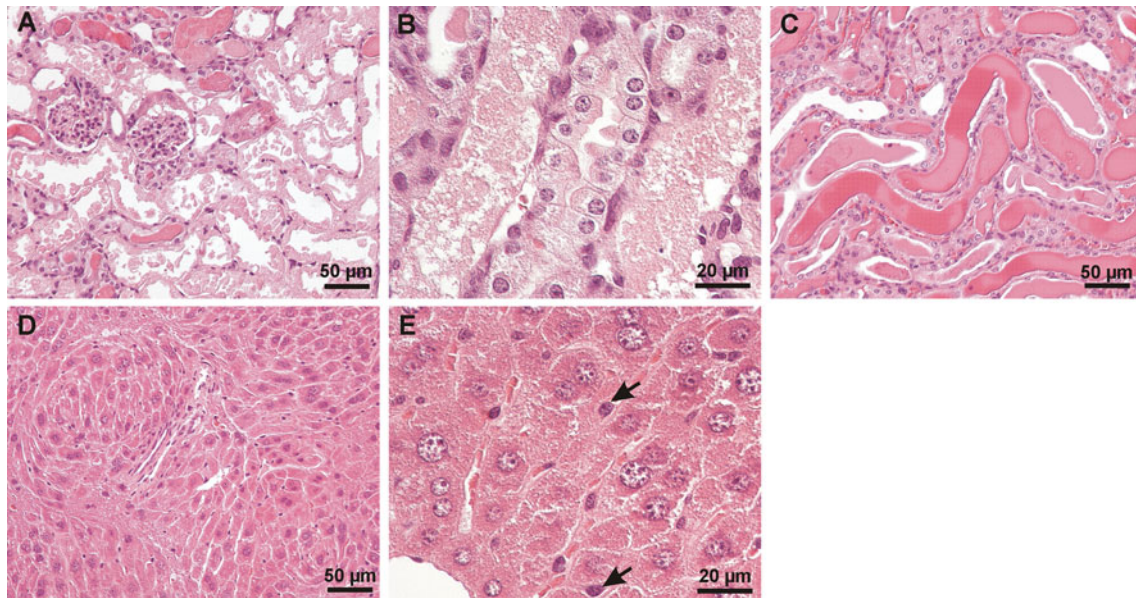


Fig. 2 Renal and hepatic lesions in FVB/N mice killed 6–9 days after a single intraperitoneal administration of SMF (250 mg/kg). All sections were stained with haematoxylin and eosin. **a** acute tubular necrosis in kidney cortex; **b** high-magnification view of necrotic

tubules; **c** protein casts in the outer strip of the renal medulla; **d** nodular hyperplasia and disarray of hepatic architecture; **e** acute centrilobular necrosis of liver—note the swollen Kupffer cells (*arrows*) and the absence of reactive leucocytic infiltrates

colon). In six of ten animals >75% of the tubules in the kidney cortex had undergone acute necrosis (Fig. 2a, b). Acute necrosis was more pronounced in proximal tubules, but also occurred in distal tubules to a lesser extent. Massive formation of proteinaceous casts was observed in the outer and inner stripe of the renal medulla as well as in the collecting ducts (Fig. 2c). Glomerular lesions were mild and involved focal necrosis of parietal cells of the Bowman's capsule. Mild granulocytic infiltrates were present in some glomeruli.

Hepatic lesions were present in all SMF-treated animals. There was scattered acute necrosis of hepatocytes. In some cases, clusters of necrotic cells were located in centrilobular areas (Fig. 2e), whereas in most cases, the necrotic cells were single cells scattered throughout the lobule. Three animals showed nodular hyperplasia (Fig. 2d). Pleomorphism of hepatocytes and cellular hyperplasia was found in four animals. Swelling of Kupffer cells was observed in all animals (Fig. 2e). Focal fibrosis, fibrin exudates along with infiltrates of macrophages and lymphocytes were present in the overlaying liver serosa of five animals. Three animals showed focal coagulation necrosis of hepatocytes adjacent to the organ surface. None of these histological anomalies (summarized in Table 1) was observed in control animals.

Experiment 2 (two treatments with 31.25–125 mg SMF/kg)

After the unexpected high toxicity in the initial experiment, we tested several lower dosages in relatively small groups

of animals. Body condition was not altered in any SMF treatment group compared to controls through the time of killing, 8 weeks after the second treatment. Hepatomegaly was present in six animals (in 2 of 4 mice at the high dosage, 3 of 4 mice at the middle dosage and 1 of 4 mice at the low dosage). Likewise, adhesions of liver to the diaphragm or the right kidney were observed (in 3 high-dosage and all 4 mid-dosage animals).

Histological lesions observed in experiment 2 are summarized in Table 2. Significant morphological lesions were serositis and fibrosis of the serosal surfaces of tissues that got into direct contact with the intraperitoneally administered compound (liver, stomach, intestine, kidneys and spleen). Adhesion between the hepatic and the renal serosal surface was detected in some animals. Regenerative tubules and protein casts were present in the kidneys of animals treated with 125 mg SMF/kg. No significant morphologic lesions were detected in lung, myocardium, pancreas, duodenum, colon (apart from serositis in one animal) and urinary bladder. No morphological lesions were observed in the control group.

Experiment 3 (4 treatments with 62.5 and 125 mg SMF/kg, extended observation period)

The mean body masses of mice showed no statistically significant differences at any time point or dosage compared to the negative control group [see Florian et al. (2012)].

Kidneys and livers of vehicle-control and SMF treatment groups were analysed for histopathological changes

Table 2 Histopathological lesions observed in FVB/N mice 8 weeks after two intraperitoneal treatments with SMF (31.25, 62.5 or 125 mg/kg)

Tissue, lesion	Incidence (mice with lesion/mice analysed)			
	SMF, high dosage	SMF, middle dosage	SMF, low dosage	Control
Kidney				
Fibrosis and serositis	1/4	2/4	0/4	0/2
Regenerative tubules	4/4	1/4	0/4	0/2
Proteinaceous casts	3/4	0/4	0/4	0/2
Pyelitis	2/4	2/4	1/4	0/2
Liver				
Fibrosis and serositis	4/4	2/4	0/4	0/2
Focal infiltration	3/4	0/4	0/4	0/2
Bile duct ectasia	0/4	3/4	0/4	0/2
Spleen				
Fibrosis and serositis	2/4	0/4	0/4	0/2
Stomach				
Gastritis	1/4	1/4	0/4	0/2
Oedema of mucosa	2/4	0/4	0/4	0/2
Jejunum				
Jejunitis	1/4	0/4	1/4	0/2
Serositis	3/4	0/4	0/4	0/2
Caecum				
Serositis	1/4	0/4	0/4	0/2

(Table 3; Fig. 3). All 14 animals of the high-SMF-dosage group (killed at varying times) had developed fibrosis of the hepatic surface (Fig. 3c; Table 3) and, except two animals, also of the renal serosa (Table 3). In the liver, this effect also occurred in most animals (20/35) of the low-SMF-dosage group, but not in vehicle-control animals (0/31). Proteinaceous casts in the kidneys were observed in 93% of the animals (13/14) of the high-SMF-dosage group, but only in 23% (8/35) and 16% (5/31) of the low-dosage and vehicle-control groups, respectively (Table 3). Regenerative tubules (Fig. 3a) were present up to week 14 after the last treatment with SMF in all animals of the high-dosage group (8/8), but were less common in the low-dosage subgroups (5/21) and vehicle-control subgroups (1/18) killed in same observation period (Table 3). They were absent in all three groups at later time points. Focal hyperplasia and in some cases also atypical hyperplasia (Fig. 3b) of renal tubules were detected in 2 of 10 mice treated with SMF after 20 weeks and in 8 of 10 animals after 40 weeks (Table 3).

In addition, a number of minor histological anomalies were detected in the liver of some animals. Pigment storage was noticed in 8 of 10 animals at the first observation period after SMF treatment and in a single SMF-treated

animal at a later observation point. Single-cell necrosis was sporadically observed in SMF-treated animals, whereas focal infiltrations were found in various animals of the vehicle-control and low-SMF-dosage groups, but not in the high-SMF-dosage group.

Effects of HMF in wild-type and transgenic (high-SULT) mice

Consumption of drinking water and food as well as body mass development was similar in HMF-treated (134 or 536 mg/kg/d) and control animals [see Florian et al. (2012)]. Kidneys and livers of wild-type FVB/N and transgenic FVB/N-SULT1A1/2 mice treated with HMF did not show any macroscopic changes compared to control animals. Likewise, no changes were microscopically detected in liver. Minor lesions were observed in kidney. They involved cell deaths, mononuclear infiltrates, limited areas of interstitial nephritis, tubulitis and/or tubular damage. Damage areas ranged from only few cells to areas with a maximal extension of 300 μm (Fig. 1A-B of Supplementary Material). The lesions were combined to a score. In all four experimental series (wild-type males, transgenic males, wild-type females and transgenic females), the highest median scores were observed in the high-HMF-dosage group (Fig. 4). This consistency suggests that the effects were real in all groups, although statistical significance could only be demonstrated for the wild-type female series. Our findings are similar to those reported for HMF in a 3-month gavage study in C3B6F₁ mice (NTP 2010): in that study, the incidences of minimal to mild cytoplasmic alteration of the kidney were significantly increased in males of the 188-, 375- and 750-mg/kg-dosage groups.

Additionally, we studied the expression of the proliferation marker Ki-67 in sections of renal and hepatic tissue. An increased proliferation would indicate a regenerative activity of the tissue as a result of previous lesions occurring over the course of twelve experimental weeks. However, Ki-67-immunoreactivity in kidneys was largely restricted to infiltrating mononuclear cells in areas of interstitial nephritis (Fig. 1B-C of Supplementary Material). Kidneys were semi-quantitatively scored in three grades according to the frequency of Ki-67-positive cells. The results suggest increased infiltration by proliferative cells: eight of 24 animals in the high-HMF-dosage groups showed grade-2 and/or grade-3 responses (males and females, wild-type and transgenic animals combined), whereas only six and four of 24 animals reached grade-2 responses in the other groups (low HMF dosage and negative controls, respectively) (Table 1 of Supplementary Material). In the liver, Ki-67 immunoreactivity was primarily detected in hepatocytes rather than infiltrating cells (Fig. 1D of Supplementary Material). Only in female

Table 3 Histopathological lesions observed in FVB/N mice at varying times after four intraperitoneal treatments with SMF (62.5 or 125 mg/kg)

Tissue, lesion	SMF dosage	Incidence (mice with lesion/mice analysed)					
		Week 4	Week 8	Week 14	Week 20	Week 40	Total
Kidney							
Fibrosis and serositis	None	0/7	0/7	0/7	0/7	0/3	0/31
	Low	1/7	0/7	0/7	0/7	2/7	3/35
	High	2/3	2/2 ^a	3/3	2/3	3/3	12/14
Tubular necrosis	None	0/7	0/7	0/7	0/7	0/3	0/31
	Low	1/7	0/7	0/7	0/7	0/7	1/35
	High	1/3	0/2 ^a	0/3	0/3	0/3	1/14
Regenerative tubules	None	0/7	0/7	1/7	0/7	0/3	1/31
	Low	3/7	0/7	2/7	0/7	0/7	5/35
	High	3/3	2/2 ^a	3/3	0/3	0/3	8/14
Proteinaceous casts	None	1/7	0/7	1/7	3/7	0/3	5/31
	Low	0/7	0/7	2/7	3/7	3/7	8/35
	High	3/3	2/2 ^a	3/3	3/3	2/3	13/14
Pyelitis/nephritis	None	3/7	2/7	2/7	3/7	2/3	12/31
	Low	0/7	2/7	2/7	7/7	1/7	12/35
	High	0/3	0/2 ^a	3/3	1/3	1/3	5/14
Hyperplasia	None	0/7	0/7	0/7	0/7	0/3	0/31
	Low	0/7	0/7	0/7	0/7	6/7	6/35
	High	0/3	0/2 ^a	1/3	2/3	2/3	5/14
Liver							
Focal infiltration	None	3/7	3/7	0/7	5/7	0/3	11/31
	Low	3/7	2/7	3/7	1/7	0/7	9/35
	High	0/3	0/2 ^a	0/3	0/3	0/3	0/14
Pigment storage	None	0/7	0/7	0/7	0/7	0/3	0/31
	Low	5/7	0/7	2/7	0/7	0/7	7/35
	High	3/3	0/2 ^a	0/3	0/3	1/3	4/14
Fibrosis and serositis	None	0/7	0/7	0/7	0/7	0/7	0/31
	Low	5/7	2/7	6/7	3/7	4/7	20/35
	High	3/3	2/2 ^a	3/3	3/3	3/3	14/14
Single-cell necrosis	None	0/7	0/7	0/7	0/7	0/7	0/31
	Low	0/7	2/7	1/7	0/7	0/7	3/35
	High	1/3	0/2 ^a	1/3	0/3	0/3	2/14

^a One mouse of the high-dosage 8-week group died ahead of time (3 weeks after the last treatment)

transgenic mice of the high dosage, the count of Ki-67-positive hepatocytes was significantly higher compared with the control group (Fig. 5).

Discussion

In experiments designed to study the induction of aberrant crypt foci, we detected high acute toxicity of SMF. After a single dose of 250 mg/kg, 20 of 35 mice died within 5–11 days. Ten further animals that were in extremely bad conditions were euthanized. However, all but one animals (4 of 4 in experiment 2 and 13 of 14 in experiment 3) receiving the next lower dosage (125 mg/kg) survived the treatment. Thus, the acute lethal dose for 50% of the

treated animals (LD₅₀) of SMF was above 125 mg/kg, but below 250 mg/kg. Three major target sites for SMF were detected, the serosas of organs located in the peritoneal cavity, the liver and the kidneys.

Serositis and reactive fibrosis were observed in the liver and kidneys of nearly all animals dosed at 125 or 250 mg SMF/kg. Spleen, stomach and/or intestines were also affected in some animals. Serositis and reactive fibrosis are indicative for local tissue damage after the intraperitoneal injection, probably due to the alkylating properties of SMF.

Sulpho conjugates are terminal metabolites of numerous xenobiotics and are excreted in bile or urine. If they are produced elsewhere, concentrative uptake into liver and kidney often occurs. We have shown that SMF is a substrate for human organic anion transporters (OAT) 1 and 3

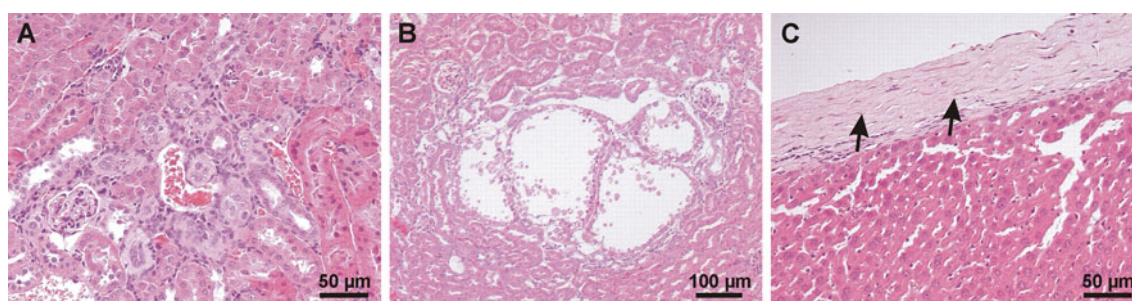
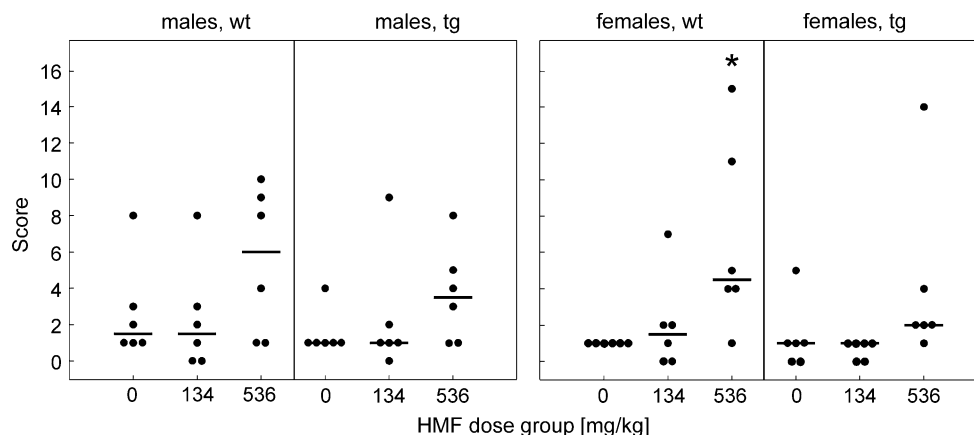


Fig. 3 Renal and hepatic lesions in FVB/N mice after four intraperitoneal treatments with SMF (125 mg/kg/treatment, with one-week intervals). All sections were stained with haematoxylin and eosin. **a** regenerative tubules 8 weeks after the last SMF

treatment; **b** hyperplasia of renal tubules 14 weeks after the last SMF treatment; **c** marked fibrosis of the hepatic serosal surface 14 weeks after the last SMF treatment—arrows mark mature collagen

Fig. 4 Pathomorphological score of the kidney of wild-type FVB/N (wt) and transgenic FVB/N-SULT1A1/2 (tg) mice receiving 0, 134 or 536 mg HMF/kg body mass/day in the drinking water for 12 weeks. Values for the individual animals (points) and their medians (lines) are presented. * $p < 0.05$ (Mann–Whitney U test compared to vehicle control)



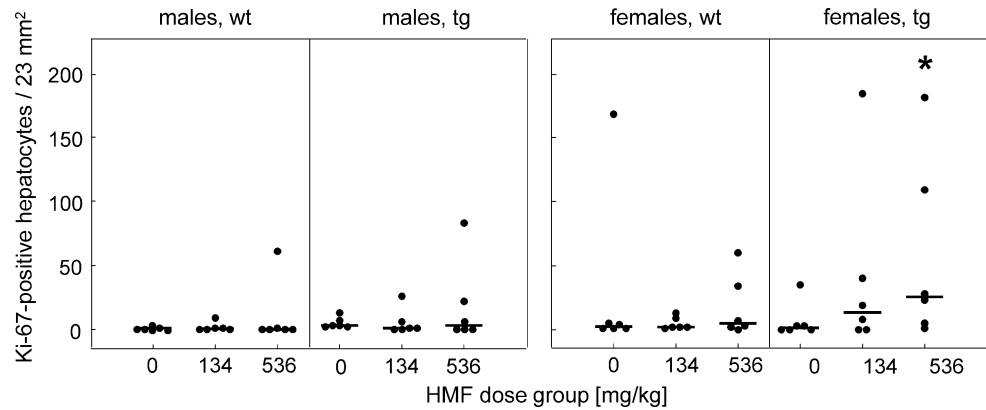
(Bakhiya et al. 2009). These transporters are expressed in renal tubules with high tissue and cell selectivity; they mediate the renal excretion of numerous anionic metabolites of drugs and other xenobiotics (Burckhardt and Burckhardt 2003). Various other anion transporters, such as OATPs (organic anion transporting peptides), MRPs (multidrug-resistance-associated proteins) and BCRP (breast cancer resistance protein), able to transport organic anions across cells, occur in liver (Chandra and Brouwer 2004) and may be involved in the uptake of SMF (e.g., when administered intraperitoneally) or its extrusion (e.g., when formed by hepatic SULTs from HMF). This biological mechanism may play a role in the hepatic and renal organotropism observed with SMF.

Acute hepatic necrosis was present in all ten animals that had received 250 mg SMF/kg and were available for analysis. Foci of hepatic hyperplasia were developed in three animals. This hyperplasia represents an early stage in hepatic tumorigenesis. Interestingly, a single intraperitoneal dose of 5-chloromethylfurfural, an uncharged analogue of SMF, induced high incidences and multiplicities of hepatomas in newborn mice (Surh et al. 1994). However, hepatic damage by SMF, apart from serositis and

fibrosis, was essentially restricted to the highest dosage level (250 mg/kg) in our study.

SMF induced the most impressive lesions in the kidneys. Nearly all proximal tubules were completely destroyed in the moribund mice euthanized in the initial experiment, in which a dosage of 250 mg SMF/kg was used. Therefore, it is probable that renal failure was the cause of the many spontaneous deaths in this group of animals. In the subsequent experiments, lower dosages were used and histological analyses were conducted at later stages (4–40 weeks after the last treatment). Although tubular necrosis was still observed in individual animals in week 4, sequelae dominated the histological lesions observed in this experiment. Regeneration of renal tubules was found until week 14, whereas proteinaceous casts were still present in week 40 after the last SMF treatment. Atypical hyperplasia was absent in control animals, but was seen with a lag period in SMF-treated animals. Its incidence in SMF-treated animals increased to 80% (8 of 10 mice) by the latest study time, 40 weeks after treatment. Further studies would be required to see whether these hyperplasias progress to clear cell carcinomas.

Fig. 5 Count of Ki-67-positive hepatocytes in liver sections of wild-type FVB/N (wt) and transgenic FVB/N-SULT1A1/2 (tg) mice receiving 0, 134 or 536 mg HMF/kg body mass/day in the drinking water for 12 weeks. Values for the individual animals (*points*) and their medians (*lines*) are presented. * $p < 0.05$ (Mann–Whitney *U* test compared to vehicle control)



Humans are not directly exposed to SMF, but this short-lived reactive molecule may be metabolically formed from a common heat-induced food constituent, HMF. Therefore, the question arises whether the SMF formed during this biotransformation can reach levels sufficient to evoke adverse effects in humans. This is difficult to test in conventional mouse and rat models due to species-dependent differences in the *SULT*. Among the human, rat and mouse *SULT* forms tested, human *SULT1A1* demonstrated the highest catalytic efficiency (Glatt and Sommer 2006; Sommer 2006). Moreover, this form is distributed at high levels in many different tissues (Teubner et al. 2007). Recently, we constructed a series of mouse lines harbouring the human *SULT1A1-SULT1A2* gene cluster, including large flanking sequences containing regulatory elements (Dobbernack et al. 2011). The lines differed in the number of copies of the *SULT1A1-SULT1A2* gene cluster integrated into the mouse genome. To be on the safe side, we used the line with the highest copy number (approximately 43 in hemizygous animals) in the present study. A copy number polymorphism of the *SULT1A1* gene occurs in humans, with up to five copies being detected in some subjects (Hebbring et al. 2007). Liver, kidney and colon mucosa of these animals express clearly higher HMF *SULT* activity than corresponding tissues of wild-type mice [“[Animals](#)” section; further information in Florian et al. (2012)]. Nevertheless, HMF-administered at high dosages via the drinking water for 12 weeks—only showed mild toxic effects to both wild-type and transgenic mice, primarily in kidneys; as the effects were similar in both mouse strains, it is not clear whether SMF was causally involved in the toxicity. As outlined in preceding sections, SMF is actively taken up from blood into proximal tubule cells by organic anion transporters. Therefore, blood levels of SMF will be important. Nearly 500 ppm of the HMF dose reaches the circulation as SMF in male wild-type FVB/N mice (Monien et al. 2011). As a first estimate, this level may be approximately 12-fold higher (6,000 ppm) in

transgenic animals, which express 12-fold higher HMF *SULT* activity in the liver, a large organ with high xenobiotic-metabolizing activities. At a dosage of 536 mg (4.3 mmol) HMF/kg, this would be 5.8 mg (25.8 μ mol) SMF/kg, which is less than a dosage of SMF [31.25 mg (140 μ mol)/kg] that did not produce detectable toxicity in our study. In this comparison, it was not taken into account that HMF was administered for 84 days, whereas SMF was only given once in the mentioned experiment (experiment 1). However, it is also evident from our experiments that splitting the dosage of SMF into a number of lower dosages given at separate times strongly reduces its toxicity. A dosage of 250 mg (1.1 mmol) SMF/kg given at once was lethal for most animals in experiment 1. When the same total dosage was given over a period of 3 weeks (4 treatments with 62.5 mg/kg/treatment at one-week intervals) in experiment 3, only relatively mild toxicity was noticed.

In the present study, a dosage of HMF of 536 mg/kg/d was required to produce some toxic effects. This dosage is far above the highest estimate for the average daily intake in humans, 150 mg per person (Ulbricht et al. 1984), equivalent to 2.1 mg/kg for a person of 70 kg body mass. Toxic effects at this high dosage of HMF were mild even in mice showing high HMF *SULT* activity [approximately 5-fold higher in liver compared to human samples (Florian et al. 2012)]; taken together, these data suggest that the relatively high HMF *SULT* activity in humans, compared to conventional animal models, does not pose a significant risk for nephro- and hepatotoxic effects of HMF ingested from food.

What remains to be studied is a possible genotoxic activity of HMF and SMF in vivo and its role in the carcinogenic effects observed with HMF. Recently, Durling et al. (2009) reported that HMF induced DNA damage, as assessed by the comet assay, in various mammalian cell lines in culture. This effect was independent of the presence of HMF *SULT* activity or h*SULT1A1* expression. However, the minimal concentration for a positive test was

extremely high [25 mM in control V79 and V79-hP-PST cells (expressing hSULT1A1), 100 mM in the other cell lines used], far above the K_m of hSULT1A1 for HMF [2.5 mM (Glatt and Sommer 2006)]. On the contrary, HMF showed another genotoxic effect, the induction of sister chromatid exchanges, even at 19.8 μ M in hSULT1A1-expressing V79 cells; this effect was clearly dependent on the expression of hSULT1A1 (Glatt et al. 2005). Likewise, HMF induced gene mutations (Glatt and Sommer 2006) and formed DNA adducts, as detected by UPLC-MS/MS analysis (B. Monien, G. Barknowitz and H. R. Glatt, manuscript in preparation) in V79-hP-PST, but not in parental V79 cells. In this context, it is interesting to know that HMF induced hepatic tumours in female, but not male, mice. This sex specificity correlates with a markedly (4.7-fold) higher hepatic HMF SULT activity in female, compared to male, wild-type mice (“Animals” section). The chief target tissues were the same in the NTP study on HMF (NTP 2010) and in the present study on SMF: liver and kidneys, but the order of the strength of the effects was reverse. In the NTP study, liver was the site of tumorigenesis, with mild microscopic changes being observed in kidneys in the 3-month study; in contrast, SMF destroyed the tubules, but only showed moderate toxicity to liver in our study. However, the toxicokinetic situation is different between the studies. After HMF administration, SMF is primarily formed in the liver and has to be exported into the circulation to produce effects in other tissues; in contrast, uptake of SMF into liver cells is required to induce hepatotoxic effects after the direct administration of the metabolite SMF.

Interestingly, furfuryl alcohol, a congener of HMF lacking the aldehyde group in 2 position, showed some evidence of carcinogenic activity in an inhalation study based on increased incidences of renal tubule neoplasms in male B6C3F₁ mice (NTP 1999). Thus, furfuryl sulphate, a mutagenic metabolite of furfuryl alcohol (Glatt et al. 2012), may be a better substrate for hepatic export systems and/or renal import systems. Moreover, furfuryl alcohol indicated some carcinogenic activity in the mucosa of the nasal cavity of mice and rats (NTP 1999), another tissue with high SULT activity (Tamura et al. 1997). Finally, furfuryl alcohol formed the same benzylic DNA adducts in mouse tissues, as are formed by furfuryl sulphate in cell-free systems (Monien et al. 2011). Altogether, a possible role of sulphation in the carcinogenic effects observed in animal models with hydroxymethylated furan derivatives deserves further investigations.

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Conflict of interest Authors declare not having any financial or personal interest, nor having an association with any individuals or organizations that could have influenced inappropriately the submitted work.

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