RESEARCH ARTICLE

Study of 5-hydroxymethylfurfural and its metabolite 5-sulfooxymethylfurfural on induction of colonic aberrant crypt foci in wild-type mice and transgenic mice expressing human sulfotransferases 1A1 and 1A2

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Scope: It was reported that the Maillard product 5-hydroxymethylfurfural (HMF) initiates and promotes aberrant crypt foci (ACF) in rat colon. We studied whether 5-sulfooxymethylfurfural (SMF), an electrophilic and mutagenic metabolite of HMF, is able to induce ACF in two murine models.

Methods and results: In the first model, FVB/N mice received four intraperitoneal administrations of SMF (62.5 or 125 mg/kg) or azoxymethane (10 mg/kg). Animals were killed 4–40 weeks after the last treatment. A total of 1064 ACF and five adenocarcinomas were detected in the azoxymethane-treated groups (20 animals), but none in the negative control and SMF-treated groups (35 and 50 animals, respectively). In the second model, HMF was administered via drinking water to wild-type FVB/N mice and transgenic mice carrying several copies of human sulfotransferase (SULT) 1A1 and 1A2 genes. HMF SULT activity was clearly elevated in cytosolic fractions of colon mucosa, liver and kidney of transgenic animals compared to wild-type mice and humans. The animals (six per group) received 134 and 536 mg HMF/kg/day for 12 weeks. HMF did not induce any ACF either in wild-type or transgenic animals.

Conclusion: We found no evidence for an induction of ACF by HMF or its metabolite SMF in extensive studies in mice.

Keywords:
Aberrant crypt foci / Human sulfotransferases 1A1 and 1A2 / 5-Hydroxymethyl furfural / 5-Sulfooxymethylfurfural

1 Introduction

One of the numerous compounds resulting from the heating of foods is 5-hydroxymethylfurfural (HMF). It is formed when reducing hexose moieties are heated in the presence of amino acids or proteins (Maillard reaction) [1] and via acid-catalyzed thermal dehydration of fructose, sucrose and glucose [2]. Particularly, high levels of HMF have been detected in caramel products (up to 9.5 g/kg), dried fruit (especially plums), old Port and Madeira wines and balsamic vinegar [3–5]. Bread and heat-sterilised milk contain lower levels of HMF, but are important sources for HMF intake, as they are consumed in high quantities. Likewise, coffee is a major source of HMF for heavy coffee drinkers [4, 5]. Older estimates of the mean daily intake of HMF from food are in the range of 30–150 mg per person [6, 7]. Estimates from newer studies from Spain [8], Norway [5] and Germany [9] are somewhat lower. However, HMF has also been detected in sterilised glucose solutions used for parenteral nutrition [10], and in cigarette smoke condensate [11, 12], wood smoke and smoke aromas (http://www.leffingwell.com/smoke.htm).

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Abbreviations: ACF, aberrant crypt foci; **HMF**, 5-hydroxymethyl furfural; **LD50**, lethal dose for 50% of the treated animals; **SMF**, 5-sulfooxymethylfurfural; **SULT**, sulfotransferase

The incidence of colorectal carcinomas varies up to 20-fold between different countries and shows pronounced temporal changes, implying an important role of lifestyle [13]. Yet, only a very small number of chemicals are known to induce this kind of neoplasia in rodents. Most often, 1,2-dimethylhydrazine or its metabolite azoxymethane, compounds lacking food relevance, are used to induce colorectal carcinomas in mice or rats. Some heterocyclic aromatic amines, in particular 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), are the major food-relevant carcinogens used for studying colorectal cancer in rodents, primarily in rats. However, the levels of heterocyclic amines in the human diet are low. Therefore, we were impressed by a finding that heated sucrose promotes azoxymethane-initiated aberrant crypt foci (ACF) in the colon of rats and mice [14] and in the subsequent identification of HMF as the active component [15]. In the latter study, it was also reported that HMF as such – i.e., without prior treatment of the animals with azoxymethane – may also be able to induce an increased number of ACF in the rat colon. The initiation of these lesions implies the induction of gene mutations in colon mucosa.

HMF is essentially inactive in standard in vitro genotoxicity tests [7, 16]. However, it is mutagenic in bacteria in the presence of rat liver cytosolic fraction supplemented with 3′-phosphoadenosine-5′-phosphosulfate, the cofactor for sulfotransferases (SULTs) [17, 18], and in bacterial and mammalian target cells engineered for expression of human SULT1A1 [16, 19]. Rat and human SULT convert HMF to a chemically reactive allylic ester, 5-sulfooxymethylfurfural (SMF) in cell-free systems [16, 17]. Godfrey et al. [20] found high levels of oxidised metabolites, but no sulfo conjugates, in urine of mice orally administered HMF. In contrast, we readily detected SMF in blood of mice shortly after the administration of HMF [21]. The negative result of Godfrey et al. may be due to technical reasons, as the detection of reactive intermediates is generally very difficult. Thus, SMF is hydrolysed to HMF in aqueous media. It has a half-life of 2 h in aqueous solutions at 37°C [21]. SMF, unlike HMF, forms DNA adducts in cell-free systems and is mutagenic to bacterial and mammalian cells without requiring an activating system [16, 17, 19]. SMF, administered topically to mouse skin, initiated papillomas with higher frequency than HMF, which also was active [17]. SMF has not yet been used systemically in animal studies.

In the present study, we investigated SMF for its ability to induce ACF and colon tumours. We used two murine experimental models. In the first model, the metabolite SMF was directly administered to the animals by intraperitoneal injection, rather than generated from HMF in the animal model. This approach assured high systemic exposure to SMF, as demonstrated by high renal toxicity (Bauer-Marinovic, M., Florian, S., Taugner, F., Glatt, H. R., manuscript submitted). The second model involved the administration 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 [22]. Both enzymes show high catalytic efficiency using HMF as the substrate [16]. In this model, SMF is generated within cells, so its relative level 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 [22], this model may best reflect human exposure to SMF.

2 Materials and methods

2.1 Test compounds

HMF [5-(hydroxymethyl)-2-furaldehyde, 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 synthesised in the laboratory of Dr. A. Seidel (Grosshansdorf, Germany) as described previously [21]. Azoxymethane (CAS 25843–45-2) was purchased from Sigma-Aldrich (Taufkirchen, Germany).

2.2 Animals

FVB/N mice were purchased from Harlan-Winkelmann (Borchen, Germany) and acclimatised for a period of at least 7 days before treatment. Transgenic FVB/N mice, carrying multiple copies of the human SULT1A1-SULT1A2 gene cluster in the middle region of chromosome 9, were propagated as a homozygous line in our laboratory [22]. They were bred with wild-type FVB/N mice to produce hemizygous mice for the experiments conducted (subsequently termed FVB/N-hSULT1A1/2 mice). All 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 undergoes drastic ontogenetic changes in the first weeks after birth before it reaches the relatively stable adult level (with large sex-dependent differences for some forms and tissues) at an age of approximately 5 weeks [23]. Likewise, hepatic expression of human SULT1A1 and hSULT1A2 in FVB/N-hSULT1A1/2 mice is fully developed at this age (unpublished results from our laboratory). Treatment was started when the animals had surpassed this critical phase by a few weeks, i.e., when they were 8–11 weeks old. Due to high sexual dimorphisms of SULT expression, both sexes were used when testing HMF. In contrast, the ultimate genotoxicant SMF was only investigated in male wild-type mice. During the experiments, animals were housed individually. All animal experiments were performed with permission (23–2347) of the Landesamt für Umwelt, Gesundheit und Verbraucherschutz of the State of Brandenburg.

2.3 Determination of HMF sulfation activity in subcellular tissue preparations

Cytosolic fractions were prepared from various tissues of wild-type FVB/N mice, hemizygous FVB/N-hSULT1A1/2 mice and humans as described elsewhere [24]. SULT activity towards the substrate HMF was determined by incubating 50 μL of a reaction mixture containing potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (5 mM), 3′-phosphoadenosine-5′-phosphosulfate (50 μM), HMF (2.5 mM) and cytosolic fraction (0.1–2 mg protein/mL) at 37°C for 5–10 min. In additional trials, incubation time and protein level were varied to verify the linearity of the reaction. The reaction was stopped by adding 150 μL of cold 2-propanol. After brief vortexing, the samples were centrifuged and the SMF concentration in the supernatant was analyzed by liquid chromatography-tandem mass spectrometry using an external calibration line containing eight SMF samples from 2.5–500 nM. Details of the analysis were reported elsewhere [21].

2.4 Treatment regimen

2.4.1 Experiment with SMF

Zhang et al. [15] had administered two gavages of HMF to animals with a 1-week interval between the treatments. They killed the animals 30 days after the second treatment for ACF scoring of colons. The individual dosages of 100–300 mg (0.8–2.4 mmol) HMF/kg body mass resulted in a dosage-dependent increase in the number of ACF. Basically, we followed this protocol but administered SMF intraperitoneally. This route was selected because natural exposure to this compound occurs via transformation of HMF to SMF in the body, rather than directly via the diet. Moreover, it was unlikely that the oral bioavailability of a reactive phase-2 metabolite would be adequate. SMF was dissolved in 0.9% sterile saline within 1 h of use. The solution was kept on ice up to a few minutes before injection, when it was warmed to room temperature. It was verified that less than 2% of the test compound was decomposed before injection. Control animals were treated with the vehicle saline. Young adult male FVB/N wild-type mice (11-week old, body mass approximately 30 g at the first treatment) were used for this part of the study. They received four intraperitoneal administrations of the following test compounds at one-week intervals: vehicle only (saline, n = 35), low dose of SMF (62.5 mg (0.27 mmol)/kg per treatment, $n = 35$), high dose of SMF (125 mg (0.55 mmol)/kg per treatment, highest tolerated dosage (Bauer-Marinovic, M., Florian, S., Taugner, F., Glatt, H. R., manuscript submitted), $n = 15$) or the positive control compound azoxymethane (10 mg/kg per treatment, n = 20). Subgroups of animals were sacrificed 4, 8, 14, 20 and 40 weeks after the last treatment. Numbers of animals (3–7) in the various subgroups are indicated in Table 2. Animals were randomly divided into these groups and subgroups at the beginning of the experiment. Body mass was recorded weekly. Due to development of colonic tumours, the last subgroup of azoxymethane-treated mice plus some control mice were killed earlier, after 30 rather than 40 weeks.

2.4.2 Experiment with HMF

At the beginning of our study, data from 3-week and 3-month gavage studies with HMF in mice and rats were available from the National Toxicology Program of the United States [25]. In that study, dosages of 750, 375 and 188 mg/kg body mass (five times per week) were found to be appropriate for long-term studies in mice. The highest and lowest dosages are equivalent to 536 mg/kg (4.3 mmol/kg) and 134 mg/kg (1.1 mmol/kg), respectively, if given daily rather than only five times a week. In the present study, male and female wild-type FVB/N and hemizygous FVB/N-hSULT1A1/2 animals (six 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 actual body mass and the average daily water consumption. It ranged from 600 to 890 mg/L in the 134 mg/kg dosage group and from 2.4 to 3.6 g/L in the 536 mg/kg dosage group. The drinking water was changed weekly. The stability of HMF in water over this period was verified by high-performance liquid chromatography with UV detection. Animals were killed immediately after the 12-week treatment. Negative control animals received pure water. A positive control was omitted, as we had demonstrated the high sensitivity of this mouse model in the preceding experiment with SMF.

2.5 Analysis of ACF

After the animals were killed by cervical dislocation, the colon was removed, cut open longitudinally, fixed flat between filter paper in 4% phosphate-buffered formaldehyde and stained with 0.2% methylene blue for the evaluation of ACF. Coded samples were inspected using a stereomicroscope (SZH10, Olympus, Hamburg, Germany).

2.6 Human tissue samples

Four liver samples, two colon mucosa samples and two kidney samples were obtained from Caucasian patients who underwent clinically indicated surgery, usually the resection of tumours [24, 26]. HMF SULT activity was studied in histologically normal tissue resected together with diseased tissue. This material was anonymised and used following the recommendations of the central ethical committee of the German Medical Association. In addition, we used a biopsy from colon mucosa taken from one of the authors undergoing routine colonoscopy.

Table 1. Activity of 5-hydroxymethyfurfural sulfo conjugation in cytosolic preparations of liver, kidney and colon mucosa of wild-type FVB/N mice, hemizygous transgenic FVB/N-hSULT1A1/2 mice and humans

	Activity, mean ± SD of three animals or two to four human subjects (pmol/min/mg)				
	Wild-type mice, males	Wild-type mice, females	Transgenic mice, males	Transgenic mice, females	Humans[a)]
Liver	25 ± 8	117 ± 18	304 ± 56	374 ± 23	67 ± 29
Kidney	22 ± 1	30 ± 6	214 ± 30	126 ± 24	5 ± 1
Colon mucosa	24 ± 11	33 ± 16	84 ± 26	98 ± 24	6 ± 2

a) All tissue samples studied were from males. No, or only minor, gender-specific differences in sulfotransferase activities have been reported in humans with any substrates tested [32].

3 Results

3.1 HMF SULT activities in various tissues of animal models and humans

In wild-type mice, the highest HMF SULT activity was detected in female liver (Table 1). This activity was 3.5- to 5.3-fold higher than that in male liver and in kidney and colon mucosa of both sexes. This finding is interesting, as HMF had induced hepatocellular adenomas in female mice but not in males and no carcinogenic activity was detected in other tissues in either sex [25].

Transgenic animals demonstrated clearly higher HMF SULT activities in all tissues investigated. In liver, the absolute increment was similar in both sexes, but the relative increase was higher in males than in females (12.2- vs. 3.2-fold) due to the higher control level in females. The activities in male kidney, female kidney, male colon mucosa and female colon mucosa were enhanced by factors of 9.7, 4.2, 3.5 and 3.3, respectively, by the transgenes.

HMF SULT was also determined in a few human tissue samples (Table 1). Hepatic activity values in human liver were intermediate to those found in male and female wild-type mice. Colonic and renal activities were lower in humans than in wild-type mice and much lower than in transgenic mice; the latter difference may be due to the fact that the transgenic animals harbour multiple copies of the human SULT1A1-SULT1A2 gene cluster.

3.2 Body mass development, feed and water consumption

The mean body masses of mice treated with SMF at a dosage of 125 mg/kg were marginally lower in the period 12–22 weeks after the treatment than those of the other groups (vehicle controls, 10 mg azoxymethane/kg and 62.5 mg SMF/kg) (Fig. 1). Afterwards, variations were more coincidental, as various subgroups had been sacrificed and the numbers of animals were smaller. None of the differences at any time point were statistically significant ($p > 0.05$, Mann–Whitney *U* test). HMF in the drinking water did not significantly affect body mass development over the treatment period in any group (male and female wild-type and transgenic mice) (Fig. 2).

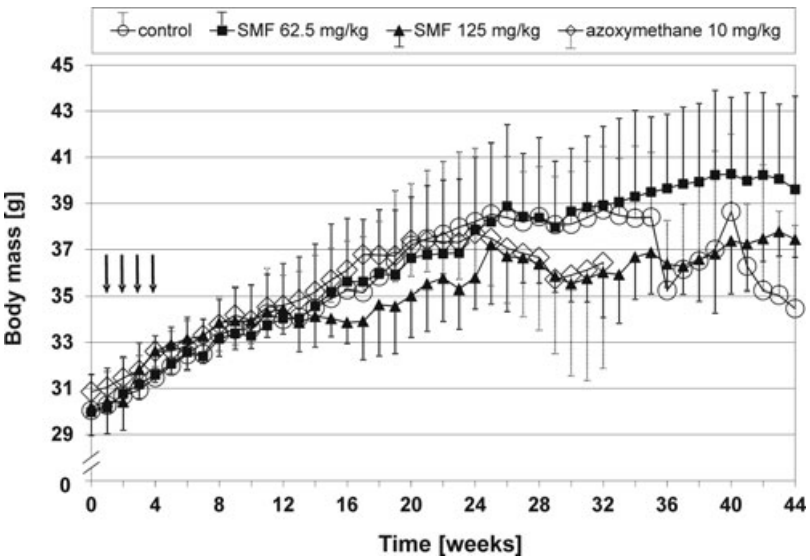

Figure 1. Body mass development of mice (males) treated four times (at the times indicated by arrows) with 62.5 mg or 125 mg 5-sulfooxymethylfurfural (SMF)/kg, 10 mg azoxymethane/kg or the vehicle (saline) only. Subgroups of animals were killed at varying times (4, 8, 14, 20, 30 and 40 weeks, see Table 2) after the last treatment. Values are means and SD of all animals living at the indicated time. The numbers of animals were small towards the end, possibly resulting in accidental deviation. Indeed, values between the groups did not differ significantly at any time point ($p > 0.05$, Mann–Whitney *U* test).

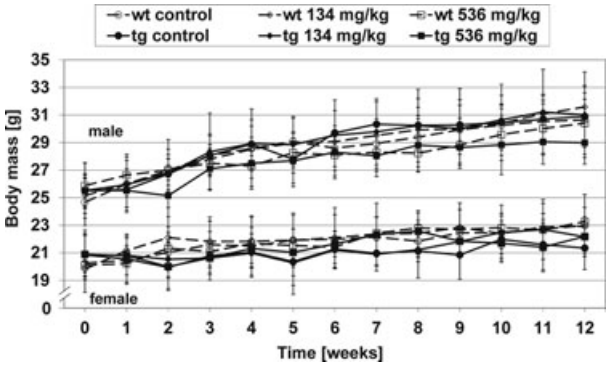

Figure 2. Body mass development of male and female wild-type FVB/N (wt) and transgenic FVB/N-SULT1A1/2 (tg) mice receiving 0, 134 or 536 mg 5-hydroxymethylfurfural (HMF)/kg body mass in the drinking water. Values are means ± SD of six animals.

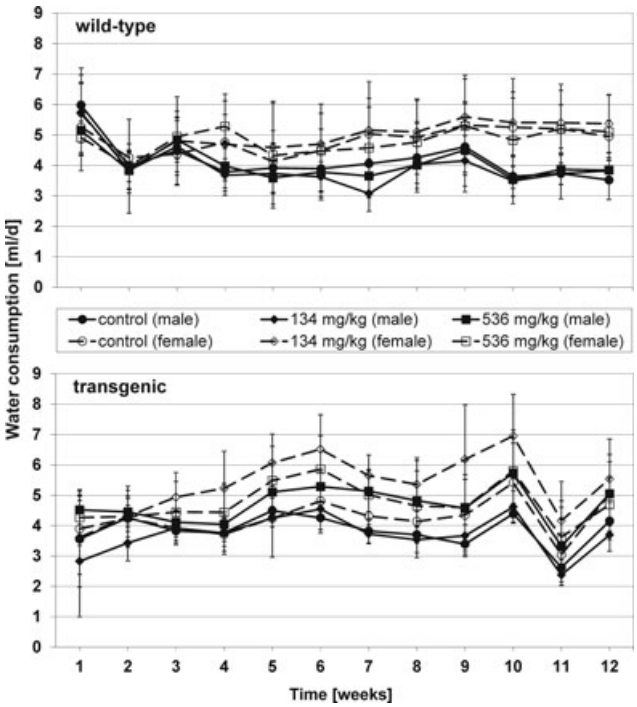

Figure 3. Water consumption of male and female wild-type FVB/N mice (upper panel) and transgenic FVB/N-SULT1A1/2 mice (lower panel) receiving 0, 134 or 536 mg 5-hydroxymethylfurfural (HMF)/kg body mass in the drinking water. Solid lines and symbols are used for males, dashed lines and open symbols represent females. Values are means ± SD of six animals.

Food consumption did not differ significantly between animals treated with the test compounds and corresponding control animals (data not shown). Consumption of drinking water was similar for the different treatment groups in wild-type mice of either sex (Fig. 3, upper panel). Among the transgenic animals, females with the low HMF dosage and males with the high HMF dosage showed a statistically insignificant tendency to higher water consumption than both other groups of the same sex (Fig. 3, lower panel).

3.3 ACF and colon tumours

In the first experiment, male FVB/N mice received four intraperitoneal treatments with SMF, azoxymethane or the vehicle (saline) at weekly intervals. Groups of animals were killed 4–40 weeks after the last treatment. The results are presented in Table 2. No ACF and no colon tumours were detected in the negative controls (in total 35 mice). In contrast, ACF were abundant in the positive control group treated with azoxymethane, at all points in time studied. The mean number of ACF per mouse increased slightly from 48 at week 4 after the last treatment to 59 at week 30. Moreover, at the latter point, three of four azoxymethane-treated mice also had developed colonic adenocarcinomas. An additional colonic adenocarcinoma was detected in one azoxymethane-treated mouse of the subgroup killed 20 weeks after the last treatment. Thus, a total of 1064 ACF and five adenocarcinomas were detected in the 20 animals of the positive control group. In contrast, none of the 50 SMF-treated animals developed ACF or adenocarcinomas.

In the second experiment, wild-type FVB/N and transgenic FVB/N-hSULT1A1/2 mice received HMF via drinking water for 12 weeks. The experiment involved 48 HMF-treated and 24 negative control animals (six animals in each group, defined by dosage level, mouse line and sex). None of these animals showed ACF or colonic tumours.

4 Discussion

It has been reported that HMF induces ACF in rat colon [15]. Most or all ACF may involve mutations. For example, Femia et al. [27] have detected K-Ras mutations in 100% (14/14) of the ACF induced in rat colon by 1,2-dimethylhydrazine. HMF itself is not mutagenic. However, a metabolite, SMF, has demonstrated mutagenic activity in bacterial and mammalian cells [16, 19, 28]. We therefore hypothesised that this metabolite may have induced the ACF in animals treated with HMF. Mice, rather than rats, were used in our study for two reasons: (i) The synthesis of SMF is demanding and has to be conducted in small batches due to its chemical reactivity, so it would have been difficult to synthesise the amounts of SMF required for a study in rats; and (ii) human SULT1A1 and SULT1A2 are particularly efficient in the activation of HMF [16]; we recently constructed a mouse line harbouring these human genes and demonstrated that the transgenic mice express the human proteins in many tissues, including colon [22], similar to the situation observed in humans [24]. However, expression levels were generally higher in the transgenic mice used than in humans, reflecting the integration of multiple copies of the human SULT1A1-SULT1A2 gene cluster into the genome of this line [22]. In the present study, we show that this genetic modification indeed leads to a clear increase in the HMF sulfation activity in subcellular preparations of colon mucosa and other tissues.

Table 2. Aberrant crypt foci and colorectal tumours observed in male FVB/N mice at varying times after four intraperitoneal treatments with 5-sulfooxymethylfurfural (SMF, 62.5 or 125 mg/kg) or azoxymethane (10 mg/kg)

	Aberrant crypt foci, mean ± SD of *n* animals (in parenthesis)			
Time, weeks after last treatment	Control	SMF, low dosage	SMF, high dosage	Azoxymethane
4	0 (7)	0 (7)	0 (3)	48 ± 7 (4)
8	0 (7)	0 (7)	0 (3)	52 ± 14 (4)
14	0 (7)	0 (7)	0 (3)	54 ± 18 (4)
20	0 (7)	0 (7)	0 (3)	53 ± 16 (4)[a)]
30	0 (4)	–[c)]	–[c)]	59 ± 15 (4)[b)]
40	0 (3)	0 (7)	0 (3)	–[c)]

a) Plus one adenocarcinoma in one animal.
b) Plus a total of four adenocarcinomas in three tumour-bearing animals.
c) Subgroup not available. Due to the appearance of tumours in azoxymethane-treated mice, the last animals of this group plus four corresponding control mice were sacrificed 30 weeks after the last treatment, whereas killing of the last SMF subgroups and the remaining control animals was postponed by ten additional weeks.

In the first experimental approach, we directly administered the metabolite SMF to mice. Although humans usually take up HMF orally, we preferred the intraperitoneal administration for the reason given in section 2.4.1. Moreover, it is not clear whether orally administered HMF reaches the colon directly via the gastrointestinal tract. In both rats and mice, HMF is rapidly absorbed with an oral bioavailability close to 100%, then swiftly metabolised and excreted in urine [29, 30]. We tested SMF up to the limits dictated by toxicity, which targeted the renal tubules (Bauer-Marinovic, M., Florian, S., Taugner, F., Glatt, H. R., manuscript submitted). The renal toxicity implies that the administered SMF was systemically available. Nevertheless, SMF did not induce a single ACF, although we used large numbers of animals and observed very strong effects with the positive control compound azoxymethane.

In the second experimental approach, mice received HMF via drinking water for 12 weeks. To study the role of SMF, we used transgenic mice expressing high levels of human SULT1A1 and 1A2 enzymes showing high catalytic activity towards HMF [16]. Human SULT1A1 and 1A2 enzymes are present at high levels in many tissues of the transgenic mice, including small and large intestine and liver [22]. Thus, if HMF reached the colonic mucosa along with the intestinal content from the luminal site, it could be activated directly in the target tissue. If it were absorbed in preceding sections of the alimentary tract, it might either be activated to SMF in other tissues and then reach colon mucosa via the circulation, or HMF may be delivered via the circulation to the colon mucosa for activation at this site. However, HMF did not induce ACF in transgenic or wild-type mice. This negative result was somewhat surprising, as the total exposure in our study (84 times 134 or 536 mg/kg) was 19–225 times higher than exposures (twice 100–300 mg/kg) that had given positive results in rats in Zhang et al. [15]. Thus, there may be pronounced species-dependent differences in the effects of HMF. It should also be noted that the background numbers of ACF varied between the experiments reported in that study and the numbers of ACF in HMF-treated animals were only slightly above background levels. It is also not clear how statistical analyses were carried out.

In this context, two other studies should be mentioned. Recently, long-term studies in rats and mice have been conducted with HMF in the National Toxicology Program of the United States [25]. HMF showed neither neoplastic nor non-neoplastic effects in the intestinal tract of either species. However, it significantly increased the incidence of hepatocellular adenomas in female mice at dosages of 188 and 375 mg/kg, where the incidence of tumours was 53% and 52%, respectively, compared to a value of 28% for the actual negative control and 22% for the mean historical negative control. No evidence of carcinogenic activity was observed in male mice and in rats. Another study was conducted in newborn Min/+ mice, which are very sensitive to spontaneous and chemically induced intestinal carcinogenesis due to hemizygosity of the Apc tumour suppressor gene. HMF increased the number of adenomas in small intestine, whereas SMF increased the number of flat ACF in the large intestine of these mice; both effects were statistically significant [31]. These positive results may be due to the high sensitivity of this special model.

In conclusion, we found no evidence for an induction of preneoplastic or neoplastic lesions by HMF or its metabolite SMF in mouse colon, whereas the positive control compound demonstrated very strong effects. Our findings are in agreement with negative results of a long-term study in mouse and rats. In that study, no neoplastic and non-neoplastic effects of HMF were observed in intestine of either species [25].

Negative results with HMF were also obtained in transgenic mice carrying multiple copies of the human SULT1A1-SULT1A2 gene cluster and expressing high HMF SULT activity in colon mucosa. Therefore, it is unlikely that humans are particularly sensitive to adverse effects of HMF in colon compared to rodent species. This conclusion is shared by authors of the Federal Institute of Risk Assessment, who recently reviewed the toxicology of HMF in food and had advanced access to the results of our study [9].

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The authors have declared no conflict of interest.

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