Brief Communication

Genotoxicity of Three Food Processing Contaminants in Transgenic Mice Expressing Human Sulfotransferases 1A1 and 1A2 as Assessed by the In Vivo Alkaline Single Cell Gel Electrophoresis Assay

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The food processing contaminants 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 5-hydroxymethylfurfural (HMF) and 2,5 dimethyl-furan (DMF) are potentially both mutagenic and carcinogenic in vitro and/or in vivo, although data on DMF is lacking. The PHIP metabolite N-hydroxy-PhIP and HMF are bioactivated by sulfotransferases (SULTs). The substrate specificity and tissue distribution of SULTs differs between species. A single oral dose of PhIP, HMF or DMF was administered to wild-type (wt) mice and mice expressing human SULT1A1/1A2 (hSULT mice). DNA damage was studied using the in vivo alkaline single cell gel electrophoresis (SCGE) assay. No effects were detected in wt mice. In the hSULT mice, PhIP and HMF exposure increased the levels of DNA damage in the liver and kidney, respectively. DMF was not found to be genotoxic. The observation of increased DNA damage in hSULT mice compared with wt mice supports the role of human SULTs in the bioactivation of N-hydroxy-PhIP and HMF in vivo. Environ. Mol. Mutagen. 56:709–714, 2015.   © 2015 The Authors. Environmental and Molecular Mutagenesis Published by Wiley Periodicals, Inc.

Key words: furan derivative; heterocyclic amine; comet assay; DNA strand break; DNA cross linking; sulfotransferase

INTRODUCTION

Numerous substances are formed during heat treatment of foods, including various genotoxic contaminants. Some contaminants are converted to genotoxicants by enzymes, and sulfotransferases (SULTs) are known to generate reactive electrophiles that can bind to DNA [Glatt, 2006]. In this study, we investigate three substances that are known to be activated, or show structural alerts for possible bioactivation by SULTs.

The well-known food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is formed in meat and fish that is prepared at high temperatures [Sinha et al., 1998]. N-hydroxy-PhIP is formed by oxidation of the exocyclic amine group by cytochrome P450 (CYP),

and this metabolite can subsequently be conjugated and bioactivated by SULTs (Fig. 1A) [Glatt, 2006]. The presence of human SULTs (hSULTs) increased the reactivity

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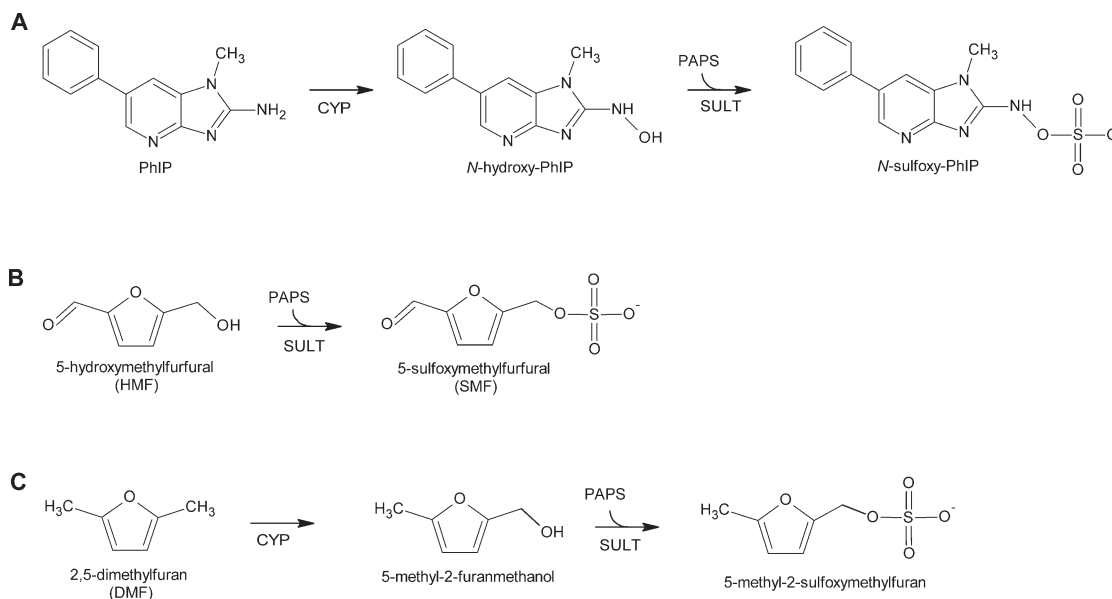
Fig. 1. The structural formulas of the test compounds and the proposed activation pathways leading to formation of reactive sulfo-conjugates catalysed by SULT in the presence of cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS). **A**: 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is transformed by cytochrome P450 (CYP) to N-hydroxy-PhIP and subsequently sulfo-conjugated by SULT. **B**: The SULT mediated transformation of HMF to SMF. **C**: Oxidation of DMF by CYP to form 5-methyl-2-furanmethanol may facilitate subsequent SULT mediated conjugation and activation. The sulfo-conjugated metabolite of DMF has not yet been detected experimentally.

of PhIP both in vitro [Muckel et al., 2002] and in vivo [Dobbernack et al., 2011; Svendsen et al. 2012].

Furan derivatives, such as 5-hydroxymethylfurfural (HMF), are also formed during food processing. HMF is present in a range of heat treated foods, especially in coffee [Husøy et al., 2008], and it is also approved as a food flavouring substance [EFSA, 2011a]. The overall mutagenic effect of HMF in conventional in vitro genotoxicity assays is low [NTP, 2010; Severin et al., 2010; Capuano and Fogliano, 2011], but positive results have been reported from the single cell gel electrophoresis (SCGE) assay [Durling et al., 2009; Severin et al., 2010]. HMF is directly activated by SULTs into 5-sulfoxymethylfurfural (SMF), which mediate genotoxic effects in vitro [Surh et al., 1994; Glatt et al., 2012] (Fig. 1B).

The less studied furan derivative 2,5-dimethylfuran (DMF) is also detected in heated foods [Fromberg et al., 2014]. It has previously been used as a food flavouring agent, although recent safety concerns have precluded its continued use [EFSA, 2011b]. Several Ames tests of DMF were negative, but formation of micronuclei has been reported in mammalian cells in vitro [Fromowitz et al., 2012]. No in vivo studies exist on the genotoxicity of DMF. The chemical structure of DMF suggests a possible biotransformation via side chain oxidation and SULT mediated conjugation [JECFA, 2009] (Fig. 1C).

As there is considerable species difference in the substrate specificity and tissue distribution of SULTs [Glatt, 2000], conventional rodent genotoxicity tests may not be appropriate to predict the human health hazards of sub-stances bioactivated by SULTs. The aim of the present study was to examine the effect of hSULT1A1/1A2 on the genotoxicity of orally administered HMF, DMF and PhIP using a transgenic mouse model containing the *hSULT1A1/1A2* gene cluster [Dobbernack et al., 2011] and the in vivo alkaline SCGE assay.
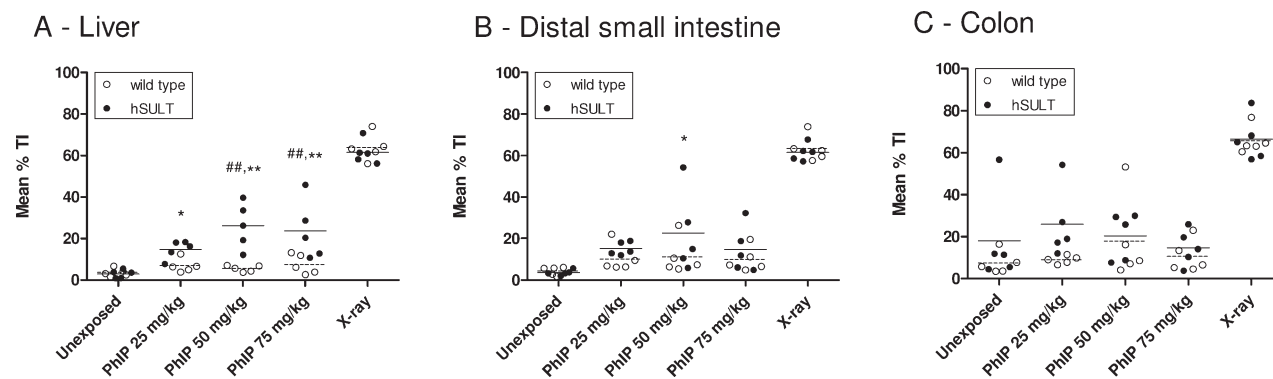
METHODS

Chemicals

PhIP-HCl (CAS no. 105650-23-5, >98% purity) from Wako (Osaka, Japan) was dissolved in saline (pH 3.5). HMF (CAS no. 67-47-0, 99% purity) and DMF (CAS no. 625-86-5, 99% purity) from Sigma Aldrich (St. Louis, MO) were dissolved in 0.9% saline or diluted in corn oil, respectively. NuSieve low melting point agarose and GelBond® films for the SCGE were both from Lonza (Rockland, ME). SYBR® Gold Nucleic Acid Gel Stain (10,000 × concentrate in DMSO) was obtained from Life Technologies™ (Carlsbad, CA).

Animals and Housing

FVB/N (FVB) mice expressing hSULT1A1/1A2 (hSULT mice, termed tg1 in the original publication), were constructed at the German Institute of Human Nutrition [Dobbernack et al., 2011]. The hemizygous hSULT mouse expresses high levels of hSULT1A1 in the liver, kidney, lung and intestines, and lower levels in other tissues [Dobbernack et al., 2011]. For the experiments with PhIP and HMF, male FVB wt mice purchased from the Jackson Laboratories (Bar Harbour, ME) and homozygous hSULT males of 9–11 weeks of age were used. In the DMF experiment, both the FVB wt males and the hSULT males had been bred at the NIPH, and were 8–16 weeks old. Animals were housed in plastic cages on Nestpak Aspen 4HK bedding (Datesand, Manchester, UK) with a 12 hr light/dark cycle. They were given free access to feed

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A - Liver

B - Distal small intestine

C - Colon

Fig. 2. DNA lesions measured as % tail intensity (% TI) in the (A) liver, (B) distal small intestine, and (C) colon of wild type and human SULT1A1/1A2 (hSULT mice) 3.5 hr after oral administration of PhIP detected by the alkaline SCGE assay. Positive assay controls (X-ray) were irrradiated with 10Gy. Each circle represents data from one mouse. Dashed (---) and solid lines (—) indicate the mean values of the wild type and hSULT mice, respectively. Statistically significant differences between the mouse lines at the same exposure level are indicated by hash signs: $P \leq 0.001$ (##). Significant differences between exposed hSULT mice and saline treated controls are indicated by asterisks: $P \leq 0.05$ (*) and $P \leq 0.001$ (**).

(RM1 Maintenance, Special Diets Services, Witham, UK) and tap water. Male littermates were cohoused until one day prior to the experiment, when they were placed in individual cages. The experiments were carried out in conformity with the laws and regulations for experiments with live animals in Norway, and were approved by the Norwegian Animal Research Authority.

Treatment, Harvest of Organs, and Processing of Samples

A single dose of test compound or vehicle was administered by oral gavage, 10 ml/kg body weight (bw). Animals received one of three dose levels of PhIP (25, 50, or 75 mg/kg bw, $n = 5$), HMF (400, 900, or 1300 mg/kg bw, $n = 5$) or DMF (75, 150, or 300 mg/kg bw, $n = 4$), and were sacrificed 3.5, 1, or 2 hr after exposure, respectively. Time points for sacrifice were based on the expected rates of metabolism.

Based on pilot experiments, the organs expected to accumulate the highest level of DNA damage for each test compound were selected for analysis. The liver, distal small intestine, and colon were analyzed from PhIP treated animals, while liver, kidney, and colon were chosen for HMF and DMF. Liver samples and kidneys were rinsed and submerged in ice-cold Merchant's buffer (14 mM NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 10 mM Na_2EDTA, pH 7.4), cut roughly, and then squeezed through a fine metal grid. Small intestine and colon were flushed with ice cold Merchant's buffer and cut open longitudinally. The outer layer of the luminal side was collected using a scalpel and transferred to Merchant's buffer on ice. Tissue suspensions from all organs were washed twice in Merchant's buffer and filtered through a 100 μm nylon mesh, with centrifugation at $300g$ for 5 min in between. Finally, cells were resuspended in Merchant's buffer to an approximate density of 10^6 cells/ml.

Aliquots (200 μl) of each cell suspension were irradiated with X-rays (10 Gy) delivered by a PXI XRAD225 unit (225 KeV, 13 mA). Radiation was filtered through 0.5 mm copper. The dose rate, as measured with Fricke's chemical dosimetry, was 3.07 Gy/min. Irradiation was performed to identify DNA interstrand crosslinks and also served as a positive assay control.

The *In Vivo* Alkaline SCGE Assay

The SCGE assay protocol has been described by Gutzkow et al. [2013], and was performed with minor modifications as follows; Gels of 4 μl were placed in quadruples onto a GelBond® film, 96 gels on each film,

and lysed overnight. The experiment was designed to include treatment of DNA with a lesion-specific endonuclease to detect a broader range of lesions; hence, two extra steps were included involving incubation of samples in a cold enzyme buffer and further incubation at 37°C in the same buffer supplemented with bovine serum albumin (negative control for endonuclease). Preliminary experiments indicated formation of endonuclease sensitive lesions following HMF exposure. In spite of this, variable and insignificant increases were observed with the enzyme in the main experiment; there were indications that the enzyme preparation was inactive. These data were therefore omitted, and the presented results exclusively represent DNA strand breaks and alkali labile sites. Unwinding of DNA was performed as described [Gutzkow et al., 2013], and the alkaline electrophoresis was conducted at 25 V total voltage (0.7–0.9 V/cm on the platform area) with circulation for 20 min. Neutralization, fixation, and staining with SYBR® Gold was performed as described [Gutzkow et al., 2013]. DNA was examined using a 20× magnification lens in an Olympus BX51 microscope (light source: X-Cite® 120Q from Excelitas Technologies, Waltham, MA; camera: A312f-VIS, from BASLER, Ahrensburg, Germany). Scoring was performed using the Comet Assay IV software (Perceptive Instruments, Bury St. Edmunds, UK). The fluorescence intensity of the tail relative to the total intensity of the head (% tail intensity, TI) was used as a measure for DNA damage.

Statistical Analysis

The mean of the median % TI from four technical replicate gels (30 nuclei per gel) was calculated for each sample, as suggested by Bright et al. [2011]. Differences between the groups were evaluated with a two-way analysis of variance, applying the Holm-Šídák method for pairwise multiple comparison procedures, in SigmaPlot version 12.0 (Systat Software GmbH, Erkrath, Germany). A P-value of ≤ 0.05 was considered significant.

RESULTS

DNA Single-Strand Breaks and Alkali Labile Sites Induced by PhIP

No increase in DNA damage was detected in any of the investigated tissues in wt mice 3.5 hr after PhIP exposure (Fig. 2). Compared with untreated hSULT mice (2.9% TI), a 5.0, 8.9, and 8.0-fold increase in % TI was observed in

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A - Liver
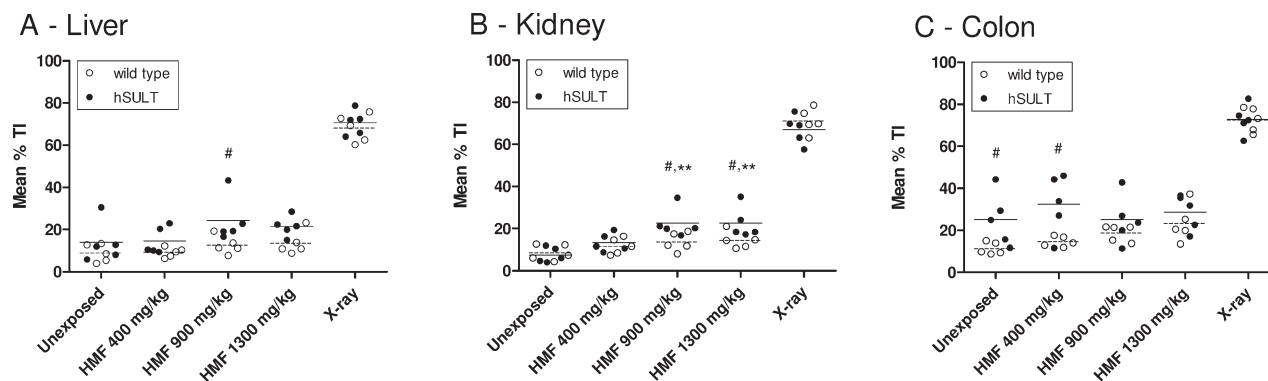
B - Kidney

C - Colon

Fig. 3. DNA lesions measured as % tail intensity (% TI) in the (A) liver, (B) kidney, and (C) colon of wild type and human SULT1A1/1A2 (hSULT mice) 1 hr after oral administration of HMF detected by the alkaline SCGE assay. Positive assay controls (X-ray) were irradiated with 10Gy. Each circle represents data from one mouse. Dashed (---) and solid lines (—) indicate the mean values of the wild type and hSULT mice, respectively. The lines indicating mean values of irradiated assay controls of colonic DNA are barely distinguishable due to similar % TI. Statistically significant differences between the mouse lines at the same exposure level are indicated by hash signs: $P \leq 0.05$ (#). Significant differences between exposed hSULT mice and saline treated controls are indicated by asterisks: $P \leq 0.001$ (**).

the DNA from the liver of hSULT mice exposed to 25, 50, and 75 mg PhIP/kg bw, respectively, (Fig. 2A). This effect appears to be dose-related, although a plateau is reached at 50 mg PhIP/kg bw. Compared with untreated hSULT mice (3.5% TI), there were also increased levels of DNA damage in the small intestine of hSULT mice exposed to 25 (4.3-fold), 50 (6.4-fold), and 75 (4.2-fold) mg PhIP/kg bw, reaching statistical significance only at the middle dose (Fig. 2B). No significant effects were detected in the colon of hSULT mice (Fig. 2C).

DNA Single-Strand Breaks and Alkali Labile Sites Induced by HMF

Oral administration of HMF did not lead to detection of increased levels of DNA damage in tissues of wt mice 1 hr after exposure (Fig. 3). In hSULT mice however, a single dose of either 900 or 1300 mg HMF/kg bw each tripled the % TI in the kidneys compared with untreated hSULT mice (7.4% TI). The minor increase in renal DNA damage following exposure to 400 mg HMF/kg bw did not reach statistical significance (1.9-fold, $P = 0.054$; Fig. 3B). Results obtained from hepatic and colonic DNA showed no significant increase in DNA damage following HMF exposure. However, there were some moderate differences between the wt and hSULT mice, namely increased DNA damage in the liver of hSULT mice treated with 900 mg HMF/kg bw (1.9-fold, Fig. 3B) and in the colon of hSULT mice given vehicle or 400 mg HMF/kg bw (both 2.2-fold, Fig. 3C) compared with equally treated wt mice.

DNA Single-Strand Breaks and Alkali Labile Sites Induced by DMF

No increased levels of DNA damage were detected in wt mice 2 hr after oral administration of DMF, nor in the liver of hSULT mice (Fig. 4). A moderate but significant 2.1-fold increase in the % TI was detected in DNA from the kidney of hSULT mice receiving 150 mg DMF/kg bw compared with untreated hSULT mice (13.2% TI). A similar tendency was observed at the highest dose of 300 mg DMF/kg bw in the same organ (1.8-fold increase), but this was not significant ($P = 0.094$; Fig. 4B). In the colon, exposure to 150 mg DMF/kg bw induced a significant 1.9-fold increase in % TI, and the lowest and the highest DMF doses also showed nonsignificant tendencies of increase ($P = 0.075$ and $P = 0.090$, respectively). There was also a significant difference in % TI in colonic DNA between the untreated animals of the two genotypes (Fig. 4C). None of the observed increases in % TI after DMF exposure were clearly dose-related, and the increases were moderate in magnitude.

Irradiation of DNA Samples for Positive Assay Control and Detection of Interstrand Crosslinks

The % TIs of the positive SCGE assay controls ranged between 65 and 80% (Figs. 2–4). No significant reduction in % TI was detected after irradiation of DNA from any organ of exposed mice, which indicate absence of interstrand crosslinks (Supporting Information Table SI).

DISCUSSION

In this study, we examined the effects of hSULT1A1/1A2 on the genotoxicity of the food processing contaminants PhIP, HMF, and DMF in mouse tissues after a single oral exposure, using the alkaline SCGE assay. In the transgenic hSULT mice, some increased levels of DNA damage were observed following exposure to all three compounds, although the magnitude of effect differed, and a dose-response relationship was only observed for PhIP.

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A - Liver
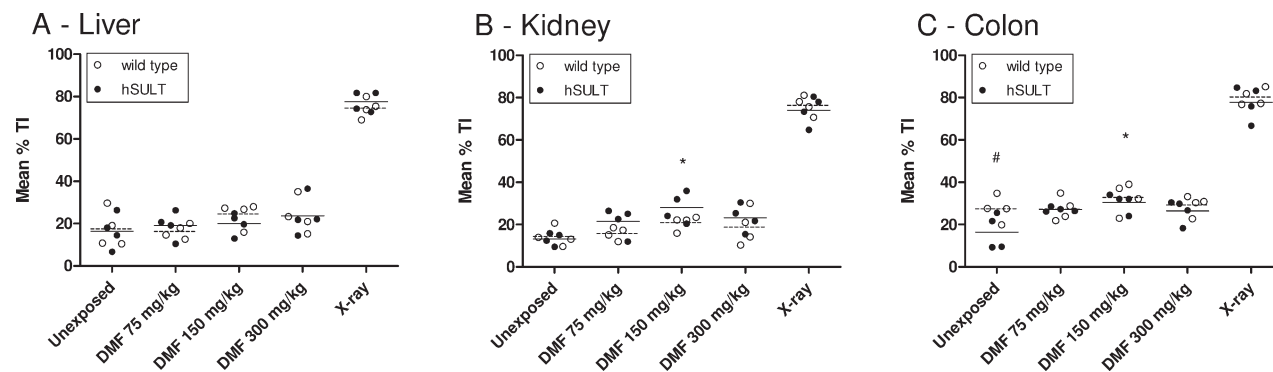
B - Kidney

C - Colon

Fig. 4. DNA lesions measured as % tail intensity (% TI) in the (A) liver, (B) kidney, and (C) colon of wild type and human SULT1A1/1A2 (hSULT) mice 2 hr after oral administration of DMF detected by the alkaline SCGE assay. Positive assay controls (X-ray) were irradiated with 10Gy. Each circle represents data from one mouse. Dashed (---) and solid (—) lines indicate mean the values of the wild type and hSULT mice, respectively. Statistically significant differences between the mouse lines at the same exposure level are indicated by a hash sign: $P \leq 0.05$ (#). Significant differences between exposed hSULT mice and corn oil treated control mice are indicated by an asterisk: $P \leq 0.05$ (*).

The detection of increased levels of DNA damage after oral exposure to PhIP was in line with previous in vivo SCGE studies [Sasaki et al., 1997, 1998]. Interestingly, in our study we did not detect any significant effect in PhIP exposed wt mice. This is in contrast to the results of Sasaki et al. [1997, 1998], who found increased DNA damage in multiple organs following ip injection of 40 mg PhIP/kg bw in CD-1 mice. PhIP requires CYP catalysed oxidation before it is a candidate for sulfo conjugation (Fig. 1). CYP is highly expressed in the murine liver [Martignoni et al., 2006], and the expression of hSULT1A1/1A2 in the liver of transgenic animals is twofold higher than in the small intestine, and fourfold higher than in the colon [Dobbernack et al., 2011]. This may explain why the liver seems to be a target organ for PhIP induced genotoxicity in hSULT mice. Another study with mice expressing hSULT1A1/1A2 also reported the liver as a target organ for induction of PhIP-DNA adducts [Dobbernack et al., 2011].

DNA from the kidneys of HMF exposed hSULT mice showed increased levels of DNA damage compared with that from untreated hSULT animals, but no increase was seen in kidneys of wt mice. To our knowledge, no other in vivo SCGE studies with HMF exist, however, several in vitro studies indicate an increased level of DNA damage following HMF exposure [Janzowski et al., 2000; Durling et al., 2009; Severin et al., 2010]. One of the studies used cells transfected with hSULT1A1, which showed that DNA damage induced by HMF was independent of the presence of hSULT [Durling et al., 2009]. On the contrary, another study reported HMF-DNA adducts in the same transfected cell line after HMF exposure and no increase in the parental cell line [Monien et al., 2012] supporting an important role of hSULT in the activation of HMF.

No SCGE results on DMF have previously been reported, and this is the first in vivo genotoxicity study to be published. The effects of DMF exposure on the DNA damage in kidney and colon were modest, and no effect was found in the liver.

In conclusion, hSULT1A1/1A2 manifest their role as important bioactivation enzymes for both PhIP and HMF, which highlight the need to carefully select appropriate animal and in vitro models before assessing the hazard of compounds that are possible substrates for human SULTs. This study does not provide convincing evidence for genotoxicity mediated by DMF in the in vivo SCGE assay.

ACKNOWLEDGMENTS

AUTHOR CONTRIBUTIONS

A.H.H., C.S., G.B., J.A., and T.H. designed the study. H.G. and W.M. shared their knowledge on the transgenic mouse model. A.H.H. and C.S. performed the experiment, analyzed the data prepared draft figures and tables. A.H.H. prepared the manuscript draft. A.H.H., C.S., and T.H. had complete access to the study data, and all authors approved the final manuscript.

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