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Determination of Sulfotransferase Forms Involved in the Metabolic Activation of the Genotoxicant 1-Hydroxymethylpyrene Using Bacterially Expressed Enzymes and Genetically Modified Mouse Models

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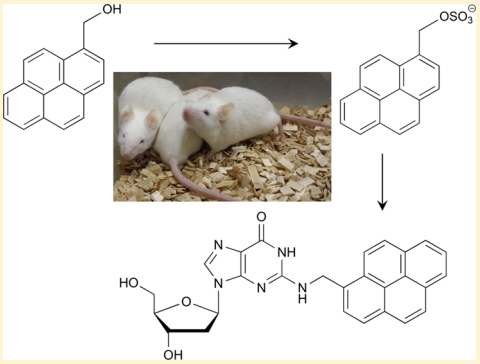
ABSTRACT: 1-Methylpyrene, a carcinogenic polycyclic aromatic hydrocarbon, forms benzylic DNA adducts, in particular N^2-(1-methylpyrenyl)-2′-deoxyguanosine, in mice and rats. It is bioactivated via 1-hydroxymethylpyrene (1-HMP) to electrophilic 1-sulfooxymethylpyrene (1-SMP). In this study, we explored the role of individual mouse sulfotransferase (SULT) forms in this activation. First, we showed that all nine mouse SULTs tested were able to activate 1-HMP to a mutagen in the his$^-$ *Salmonella typhimurium* reversion test. Some activation was even observed with Sult2a3 and Sult5a1, orphan forms for which no substrates were identified hitherto. Subsequently, we used cytosolic preparations from tissues of four mouse lines (wild-type, Sult1a1$^-$, Sult1d1$^-$, and transgenic for human SULT1A1/2) for the activation of 1-HMP in the mutagenicity assay. The most prominent impacts of the genetic SULT status were 96% decrease in hepatic activation by Sult1a1 knockout, 99% decrease in renal activation by Sult1d1 knockout, and 100-fold increase in pulmonary activation by transgenic human SULT1A1/2. Finally, we treated the various mouse lines with 1-HMP (19.3 mg/kg, intraperitoneally), and then determined 1-SMP levels in plasma and DNA adducts in tissues. Transgenic human SULT1A1/2 strongly enhanced 1-SMP plasma levels and DNA adduct formation in the liver, lung, heart, and kidney but not in the colon. Sult1a1 and Sult1d1 knockout reduced plasma 1-SMP levels as well as DNA adduct formation in some tissues (strongest effects: 97% decrease in 1-SMP and 89% decrease in hepatic adducts in Sult1a1$^-$ mice). The adduct levels detected in various tissues did not accurately reflect the activation capacity of these tissues determined *in vitro*, probably due to the distribution of the reactive metabolite 1-SMP via the circulation. In conclusion, we demonstrated that many mouse SULT forms are able to activate 1-HMP. *In vivo*, we verified a prominent role of Sult1a1 in hepatic and renal adduct formation and a smaller but unambiguous role of Sult1d1, and demonstrated the strong impact of transgenic human SULT1A1/2.



INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), a large group of environmental contaminants, can be classified into purely aromatic and alkylated congeners. Both classes of PAHs are formed by incomplete combustion of organic matter. The alkylated PAHs are preferentially generated at low combustion temperatures and by diagenesis of organic sediments. The German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK) recommended that 1-methylpyrene (structural formula in Scheme 1), as a carcinogenic representative of the alkylated PAH, be investigated along with a set of relevant nonalkylated PAHs in routine determinations.[1] 1-Methylpyrene has been found in cigarette smoke condensate.[2,3] In this matrix, the methylpyrenes (1-, 2-, and 4-isomers) are nearly 10 times as abundant as the reference PAH benzo[*a*]pyrene.[4−6] 1-Methylpyrene was also detected in car exhaust[7] and various foodstuffs, such as smoked cheese,[8] native olive oil,[9] cooking margarine,[10] grilled sausages,[11] coffee brew,[12] and as a bioaccumulated pollutant in mussels, crabs, and finfish.[13]

1-Methylpyrene is hepatocarcinogenic in newborn mice.[14] Since its structure lacks an angular terminal benzo ring, it cannot be activated to bay-region dihydrodiol-epoxides, the best established ultimate carcinogens of PAHs.[15−17] Instead, it forms benzylic DNA adducts, mainly N^2-(1-methylpyrenyl)-2′-deoxyguanosine (MPdG) in mouse liver, a target organ of its carcinogenicity, and in other mouse and rat tissues.[18] Adduct formation only occurs after the metabolic activation of 1-methylpyrene. 1-Sulfooxymethylpyrene (1-SMP, Scheme 1), an electrophilic metabolite of 1-methylpyrene in animal models,[18] forms MPdG adducts in cell-free systems and also in rat and

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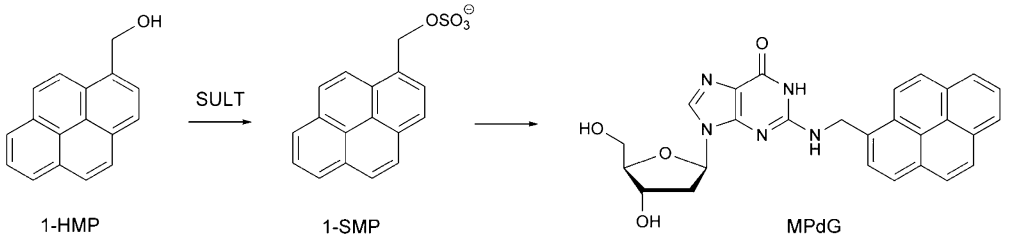
Scheme 1. Structural Formulas of 1-Hydroxymethylpyrene (1-HMP), Its Reactive Metabolite 1-Sulfooxymethylpyrene (1-SMP), and the Major Adduct, N^2-(1-Methylpyrenyl)-2′-deoxyguanosine, Formed by 1-SMP in DNA



mouse tissues after its direct administration.[19−21] 1-SMP is a direct acting mutagen in bacterial and mammalian cells.[19,20,22] Moreover, it initiated papillomas in mouse skin after topical application[20] and induced local sarcomas after subcutaneous injection in rats.[23]

Although the reactive metabolite 1-SMP is short-lived in water ($t_{1/2}$ 2.8 min at 37 °C), it could be detected readily in blood plasma of mice treated with 1-methylpyrene[18] and in rats treated with 1-hydroxymethylpyrene (1-HMP), the immediate metabolic precursor of 1-SMP.[24] This is due to the stabilization of 1-SMP by reversible binding to serum albumin. Thus, $t_{1/2}$ of 1-SMP was prolonged by a factor of 350 to 990 min, when it was incubated in rat blood serum rather than in water (at 37 °C).[25]

A number of human and rat sulfotransferases (SULTs) have been identified that are able to activate 1-HMP *in vitro*.[26−29] A small number of SULTs formed from other species, including the mouse, also showed this activity.[29] However, no information is available on the role of individual SULT forms in the activation of 1-HMP *in vivo*, apart from our finding that transgenic human SULT1A1/2 enhances plasma 1-SMP levels and MPdG adduct formation in mouse models treated with 1-methylpyrene. Information on the role of individual SULT forms is important, as they differ in their tissue distribution,[30,31] and as many forms are genetically polymorphic in humans.[32,33] The aim of the present study was to examine the role of individual mouse SULT forms in the activation of 1-HMP using *in vitro* and *in vivo* models genetically engineered in their SULT status.

EXPERIMENTAL PROCEDURES

Materials. 1-Methylpyrene was obtained from Sigma-Aldrich (Taufkirchen, Germany). 1-SMP was prepared as described by Enders et al.[34] Isotope-labeled adduct standard [$^{15}N_5$,$^{13}C_{10}$]MPdG was synthesized as described by Monien et al.[21] 3′-Phosphoadenosine-5′-phosphosulfate (PAPS) was prepared using recombinant human PAPS synthetase 1, expressed in *Escherichia coli*, and purified by preparative anion exchange HPLC. Its purity was ≥99%, as determined by HPLC with UV detection.

SULT-Expressing *Salmonella* Strains. Mouse (m) SULTs were expressed in *S. typhimurium* strain TA1538 by inserting the open reading frame of their cDNA sequences (GenBank accession numbers in Table 1) into the pKK233-2 vector. The construction of strains TA1538-mSult1a1, TA1538-mSult1b1, and TA1538-mSult1e1 has been described elsewhere.[29,35] Expression of the other mouse SULTs is reported in this study for the first time.

Mouse Lines. Wild-type FVB/N mice (subsequently termed wt mice) were purchased from Harlan (Borchen, Germany). The generation of transgenic FVB/N mice with multiple copies of the human *SULT1A1-SULT1A2* gene cluster integrated in chromosome 9 is described elsewhere.[36] The line termed tg1 in the original study was used. The homozygous transgenic line was bred with wild-type (wt) mice to generate animals with a hemizygous gene status with respect to the human transgene (subsequently termed hSULT1A1/2-tg mice).

Sult1a1$^-$ mice were constructed as described elsewhere in detail.[37] Briefly, the *Sult1a1* genomic sequence was obtained from a bacterial artificial chromosome. Exons 2−4 were replaced by a neomycin resistance cassette. This targeting vector was introduced into C57BL/6x129/Sv embryonic stem cells by homologous recombination. After selection and characterization, modified stem cells were microinjected into C57BL/6J blastocysts, which were transferred into pseudopregnant NMRI mice. High-contribution male chimeras were mated to FVB/N females. Mice showing germ line transmission of the mutant *Sult1a1* allele were backcrossed to FVB/N animals. Using marker-assisted backcrossing (Charles Rivers, Troy, MI, USA), 99.3%

Table 1. Summary of the Mutagenicity Results with 1-HMP in *S. typhimurium* Using Mouse SULTs for the Metabolic Activation

strain	GenBank accession number of the expressed sequence	SULT protein level, % of cytosolic protein	internal activation, revertants per nmol 1-HMP[a]	external activation, revertants per μg protein[b]
TA1538			<0.3	<0.03
TA1538-mSult1a1	L02331	2−4[c]	8300	100
TA1538-mSult1b1	U92076	1−2[c]	15	28
TA1538-mSult1c2	AY005 469	n.d.[f]	<0.3	16
TA1538-mSult1d1	NM_016771	10−11[e]	30	620
TA1538-mSult1e1	NM_023135	5[d]	15000	2700
TA1538-mSult2a1	L02335	n.d.	150	1500
TA1538-mSult2a2	L27121	n.d.	70	900
TA1538-mSult2a3	AK050422	n.d.	<0.3	0.2
TA1538-mSult5a1	AF026074	n.d.	<0.3	1.4

[a]Mutagenicity was determined in the recombinant strain using varying levels of 1-HMP. Representative dose−response curves are presented in Figure 1. Values represent the initial slope of the linearized dose−response curves. For negative results, a conservative limit of detection is given. Data for strains TA1538-mSult1a1 and -mSult1b1 were reported previously.[29] [b]Mutagenicity was determined in strain TA98 using fixed concentrations of 1-HMP (50 nmol in an incubation volume of 610 μL, i.e., 82 μM) and PAPS (50 μM) and varying amounts of cytosolic fraction of the recombinant strains (expressed as μg cytosolic protein). Representative protein−response curves are presented in Figure 2. Values represent the initial slope of these curves. [c]Data from Meinl et al.[29] [d]Data from Stjernschantz et al.[35] [e]Estimated in this study from Coomassie-stained gels as previously described for other SULTs.[55] [f]n.d., not determined (due to the lack of an appropriate antiserum and/or purified reference protein).

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homozygosity (except the region around the *Sult1a1* gene) in an FVB/N genetic background was reached after five generations.

Hepatic gene expression was compared in wt, hSULT1A1/2-tg, and Sult1a1$^-$ male mice (5 animals per group, at the age of 8 weeks) using Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA) according to the manufacturer's recommendations. The genetic manipulations did not lead to altered expression of other genes. This was true in particular for genes encoding other SULT forms or other xenobiotic-metabolizing enzymes.[36] (Meinl, W., and Glatt, H. R., manuscript in preparation).

Sult1d1$^-$ mice were constructed using the same procedure as that for the Sult1a1$^-$ line with the following specifications: A 20-kb fragment from the artificial chromosome RP24-571N6 (German Resource Center for Genome Research, Berlin, Germany) comprising 10 kb sequences upstream of exon 1, exons 1 to 5, and the first 1 kb of intron 5 of the *Sult1d1* gene was subcloned by Red/ET recombination into the PCR-amplified minimal vector derived from pACYC177 (New England Biolabs, Frankfurt am Main, Germany). The forward primer Sult1d1 A-ori-F 5′-GAC CTT TCT TCT AAG TAC AAT TTC ATT ATC AGG TAT GAA ATG TGG ACA TTC TCG AGT GAA GAC GAA AGG GCC TCG TG-3′ and the reverse primer Sult1d1 A-ori-R 5′-GTC ATC CAA AAA TAG ATG CAG GAG ACA CTT CAG AAG CTG TTA GAC TCA AAC TCG AGC TAG CGG AGT GTA TAC TGG C-3′ were used to amplify the ampicillin resistance and replication origin of pACYC177. *Xho*I sites (underlined) flanking the vector sequences were introduced to enable the release of the vector from the targeting sequences. Exons 2 to 4 (including intron sequences of 200 bp upstream of exon 2 and 500 bp downstream of exon 4) of *Sult1d1* within the targeting sequence were exchanged against a neomycin resistance cassette (flanked by loxP recombination sequences) from the loxP-PKG-gb2-neo-loxP plasmid (generous gift from F. Stewart, Technical University Dresden, Germany) again by using Red/ET recombination. The oligomer sequences for amplification of the neomycin resistance were as follows: Sult1d1 neo-F 5′-ACT TTA GCA TAT GCT ATT GAT ATA CAA ATG AGG TAT AGT AAT ATG CTG TGA GAA CTG TGA ATG CGC AAA-3′ and Sult1d1 neo-R 5′-AAA TCT GAT GGC TCT CAG ATT GGC ACA GCC TCA GTT GTG CTC TGG ATC CTG ATA TCA GCC ATG AGG GTT TAG TTC G-3′. Subsequent steps to yield Sult1d1$^-$ FVB/N mice were the same as those described for Sult1a1$^-$ mice (including marker-assisted backcrossing to 99.5% homozygosity except for the region around *Sult1d1*).

Mutagenicity Testing. The bacteria were grown in Nutrient Broth No. 2 (Oxoid GmbH, Wesel, Germany) at 37 °C with shaking for 8 h. Parental strains TA1538 and TA98 were grown in the absence of antibiotics. Ampicillin (50 μg/mL) was added to the growth medium for the recombinant strains. The cultures were centrifuged, suspended in medium A (1.6 g/L Bacto Nutrient Broth and 5 g/L NaCl), adjusted turbidimetrically to a titer of 1−2 × 10^9 bacteria (colony-forming units)/mL, and kept on ice. Shortly before use, they were centrifuged again and suspended at a 5-fold higher density in medium A. Mutagenicity was determined using modified versions of the liquid preincubation assay described by Maron and Ames.[38] Two versions of the assay were used.

The first version involved the testing of varying doses of 1-HMP directly in SULT-expressing strains. The bacterial suspension (100 μL) and 1-HMP (in 10 μL dimethyl sulfoxide) were added sequentially to a glass tube containing 500 μL of 100 mM $MgSO_4$. 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, 50 μM biotin, 50 μM histidine, 50 μM tryptophane, and 25 mM sodium phosphate buffer, pH 7.4) was added, and the mixture was poured onto a Petri dish containing 24 mL of minimal agar (15 mg/mL agar in Vogel-Bonner E medium with 20 mg/mL glucose). After incubation for 3 days in the dark, the colonies (his$^+$ revertants) were counted. Incubations were carried out in triplicate, except for the negative controls (3 to 9 plates).

The second version of the mutagenicity assay involved the use of a SULT-deficient target strain (TA98), a fixed dose of 1-HMP (50 nmol, or the solvent only, 10 μL of dimethyl sulfoxide), together with an external activating system, which was composed of a cytosolic fraction from recombinant strains or mouse tissues and PAPS (50 μM), the cofactor of SULTs. Unlike with various other SULT-dependent mutagens,[39] this approach is feasible with 1-HMP[40] since 1-SMP formed extracellularly is nonenzymatically converted to secondary reactive species, such as 1-chloromethylpyrene and other uncharged substitution products, that readily penetrate into the target bacteria.[19,41] Strain TA98, rather than TA1538, was used as the target strain to facilitate comparison with previous similar studies[28,40,42] and also because TA98 is nearly twice as responsive as TA1538 toward the mutagenic action of 1-SMP. Cytosolic fractions from mouse tissues and recombinant *Salmonella* strains were prepared as described elsewhere, using 150 mM KCl containing 10 mM sodium phosphate buffer (pH 7.4) as the homogenization medium.[40,43] The incubation conditions were as in version one, except that the 500 μL of $MgSO_4$ was replaced by 200 μL of solution B (150 μM PAPS, 25 mM $NaSO_4$, and 25 mM $MgCl_2$ in homogenization medium) and varying levels of cytosolic fraction completed to 500 μL with homogenization medium.

Treatment of Animals. All animal experiments were approved by the proper authorities, the Landesamt für Umwelt, Gesundheit, and Verbraucherschutz of the State of Brandenburg. Male mice (FVB/N and genetically modified lines in this background, approximately 8-week old, 5 per group) were treated intraperitoneally with 1-HMP at a dose of 83 μmol (19.3 mg) per kg body mass, using dimethyl sulfoxide (2.5 μL per g body mass) as a vehicle. Control animals only received the vehicle. Dose and route were the same as those in a previous study in rats.[24] After 7.5−120 min, the mice were anaesthetized with isoflurane. Blood (0.5−1 mL per animal) was obtained by retrobulbar puncture using heparinized Microvette CB 300 LH capillary tubes (Sarstedt, Nürnbrecht, Germany). It was immediately centrifuged (2500*g*, 10 min) to obtain the plasma. The animals were killed by cervical dislocation after taking the blood. The liver, lungs, and kidneys were removed, snap frozen, and stored at −80 °C until analysis.

Determination of 1-SMP Plasma Levels. 1-SMP plasma levels were determined as described in detail by Monien et al.[24] In brief, plasma (25 μL), immediately after its preparation, was combined with ice-cold isopropanol (75 μL) to precipitate proteins and then centrifuged at 10000*g* for 5 min. An aliquot (4 μL) of the resulting supernatant was analyzed in an Acquity ultraperformance liquid chromatography (UPLC) system with a BEH Phenyl column (1.7 μm, 2.1 × 100 mm) connected to a Quattro Premier XE tandem quadrupole mass spectrometer with an electrospray interface operated in the negative ion mode (all from Waters, Eschborn, Germany). The *m*/*z* transition 311 → 81 (formation of the protonated sulfonate ion) was used as the quantifier. The *m*/*z* transition 311 → 96 (formation of the sulfate ion radical) served as qualifier. Mouse plasma samples spiked with 1-SMP at different concentrations were used for external calibration. The limit of quantification of this method was at a plasma level of ∼10 nM 1-SMP.[24] All samples were analyzed twice, usually with <10% variation between the results of the individual measurements.

Determination of DNA Adducts by Isotope-Dilution UPLC-MS/MS. The procedure has been described in detail by Monien et al.[21] Briefly, whole tissue homogenate was extracted twice with four volumes of *n*-butanol to remove possible remnants of 1-SMP. Then, DNA was isolated from the homogenates by a standard phenol/chloroform extraction.[44] Aliquots (100 μg) of purified nucleic acids (quantified spectrometrically) were spiked with the isotope-labeled standard (2.5 pmol [$^{15}N_5$,$^{13}C_{10}$]MPdG), enzymatically digested to nucleosides, and subjected to solid-phase extraction for enrichment of the adducts as described.[21,24] The instrumentation for the UPLC-MS/MS analysis was the same as that for the 1-SMP analysis. The neutral loss of the deoxyribose (MPdG 482.3 → 366.1) was used for quantification with [$^{15}N_5$,$^{13}C_{10}$]MPdG (497.3 → 376.1) as the internal standard, while the fragmentation into the nucleoside and the 1-methylpyrene cation (*m*/*z* 215.1) was used to verify the identity of the analyte. The limit of detection (LOD) of this method was at approximately 3 MPdG and 0.6 MPdA adducts per 10^8 nucleosides.[21] All samples were analyzed twice, usually with <15% variation between the results of the individual measurements.

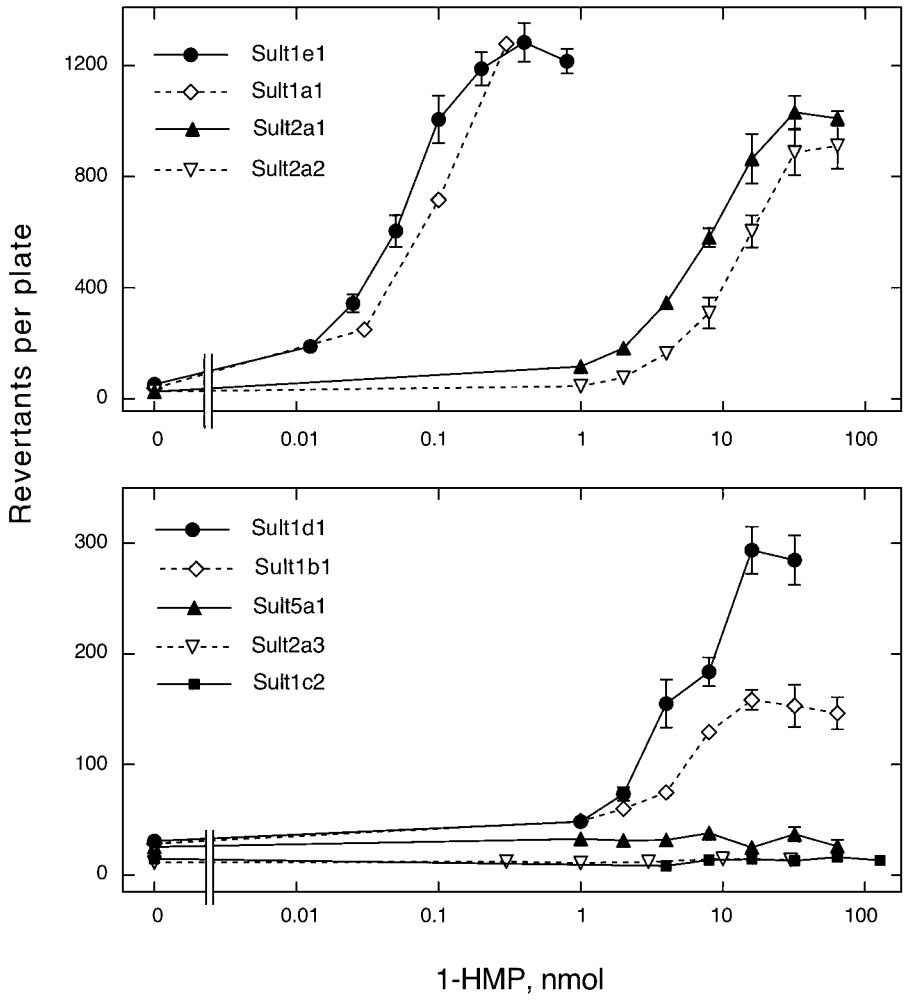

Figure 1. Activation of 1-HMP to a mutagen by mouse SULTs expressed in the target strain, *S. typhimurium* TA1538. Values are the mean ± SE of 3 plates (1-HMP-treated groups) or 3–6 plates (solvent control). Note that different ordinate scales are used in the upper and lower panels.

We recently detected that enzymatic digests of DNA prepared by phenol/chloroform extraction contains substantial levels of ribonucleosides in addition to 2′-deoxyribonucleosides (dN), despite treatment with RNase prior to the extraction.[45] Fortunately, our analytical method is specific for 2′-deoxyribonucleoside adducts, with no risk of mixing up with ribonucleoside adducts. However, the amount of DNA used in the analysis is overestimated by the spectrophotometric measurement due to the presence of RNA and its degradation products. In order to determine the actual level of DNA, we directly determined the level of 2′-deoxyguanosine in the hydrolysate using [$^{15}N_5$]2′-deoxyguanosine as the internal standard.[45] The total number of dN molecules was calculated knowing that 2′-deoxyguanosine constitutes 21% of all dN in the genomic DNA of the mouse.[46]

Determination of DNA Adducts by ^{32}P-Postlabeling Analysis. The mass spectrometric adduct analysis requires relatively large samples of DNA. Smaller quantities are sufficient for the ^{32}P-postlabeling analysis. Therefore, this method was used with some heart and colon samples, following a protocol described in detail elsewhere.[21] Briefly, DNA samples (5 μg) were digested with micrococcal nuclease and calf spleen phosphodiesterase, enriched with nuclease P1, and labeled with [γ-^{32}P]ATP in the presence of thymidine kinase. Radiolabeled adducted nucleoside 3′,5′-bisphosphates were separated by multidirectional thin-layer chromatography on polyethylenimine-cellulose plates. After the chromatography, the plates were scanned in an Instant Imager (Canberra Packard, Dreieich, Germany). The level of the dominating adduct spot (representing MPdG 3′,5′-bisphosphate) was calculated via the specific radioactivity of the [γ-^{32}P]ATP used, the relative labeling efficiency (2′-deoxyguanosine-5′-phosphate as the substrate), and the radio-counting efficiency as described previously.[21] Moreover, values were corrected for the actual content of DNA in the nucleic acid isolated, as described in the Determination of DNA Adducts by Isotope-Dilution UPLC-MS/MS section. The limit of detection of this method was at approximately 7 adducts per 10^8 nucleotides.[21]

RESULTS AND DISCUSSION

Mutagenicity of 1-HMP to *S. typhimurium* Strains Using Individual Mouse SULTs for the Bioactivation. In a first set of experiments, we tested 1-HMP on mutagenicity in various TA1538-derived strains expressing mouse SULTs. Under these conditions, six mouse SULTs demonstrated activation of 1-HMP, with TA1538-mSult1e1 and TA1538-mSult1a1 being clearly more responsive than the remaining strains (Figure 1 and Table 1). This situation is similar to that observed with human (h) SULTs: strains expressing hSULT1A1 and hSULT1E1 were most responsive, and six other human forms demonstrated some activation of 1-HMP.[29] However, orthologous mouse and human forms may substantially differ in tissue distribution. In a study conducted on the mRNA level, it was reported that *mSult1a1* is primarily expressed in the liver, large intestine, and lungs.[31] We could confirm high expression of the protein level in the liver and large intestine, whereas we could barely detect any mSult1a1

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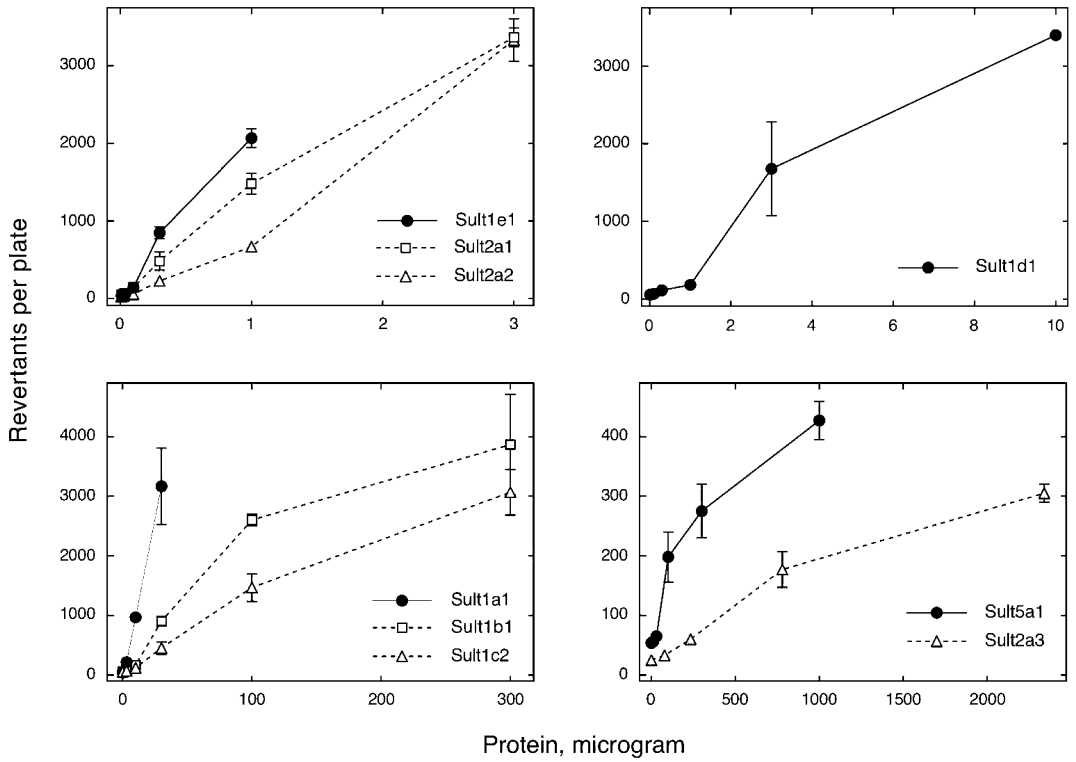

Figure 2. Activation of 1-HMP to a mutagen in *S. typhimurium* TA98 by various externally added mouse SULTs. The SULTs were expressed in *S. typhimurium*. Varying amounts of cytosolic fractions of these strains (expressed as μg total cytosolic protein in the abscissa) and the cofactor PAPS (50 μM) were used. Values are the mean ± SE of 3 plates (1-HMP-treated groups) or 3−6 plates (solvent control). Note that different axis scales are used in the various panels..

protein in the lungs (Meinl, W., and Glatt, H. R., unpublished result). On the contrary, hSULT1A1 is highly expressed as protein in numerous tissues.[36] Expression of *mSult1e1* on the mRNA level was essentially restricted to the placenta, testis, and uterus.[31]

In a second set of experiments, we used cytosolic fractions from the recombinant strains, fortified with PAPS, as an external activating system. This approach is similar to common enzyme activity assays, as it involves varying levels of the enzyme and a single, relatively high concentration of the substrate. Under these conditions, all nine mouse SULT forms demonstrated activation of 1-HMP (Figure 2 and Table 1). Clear, although weak, activation was even seen with mSult2a3 and mSult5a1, orphan forms deduced from mouse DNA (GenBank accession numbers AK050422 and AF026074) for which no substrates have been reported hitherto. The activation of 1-HMP in our experiments confirms that these cDNAs really encode SULT enzymes.

The order of mutagenic activity observed with external activation was different from that found with internal activation (Table 1). This difference is not surprising, as our assays with external and internal activations reflect different enzyme kinetic parameters, in first approximation, V_{max} and V_{max}/K_m, respectively. Moreover, bacteria may contain selective inhibitors (including competing substrates) and/or activators for some enzyme forms, and the high concentration of 1-HMP used in the assay with external activation may have led to some substrate inhibition with certain SULT forms; with other substrates, substrate inhibition was observed in particular with SULT1A1 enzymes.[47]

Furthermore, results for SULT-dependent mutagens are usually affected by the SULT expression level, although saturation was observed in some cases at high expression.[43] In a recent study, we determined expression levels of 0.25−7% SULT in the cytosolic protein for 15 recombinant strains.[29] Up to date, we were only able to determine the protein levels of four mouse SULT forms (1a1, 1b1, 1d1, and 1e1). Four other forms (1c2, 2a1, 2a2, and 2a3) were readily detected but not quantified in immunoblots using cross-reacting antisera raised against other SULTs (data not shown). Neither the antiserum nor the purified standard was available for mSult5a1. Thus, the activation of 1-HMP was the only qualitative indication for an expression of this form. Since the expression levels of five out of nine mouse SULT forms in the *Salmonella* model are unknown, any ranking of the activation efficiency is preliminary. Likewise, hardly any information is available on the SULT protein levels in mouse tissues.

Mutagenicity of 1-HMP Using Mouse Tissue Preparations for the Bioactivation. Cytosolic fractions of the liver, kidney, and colon efficiently activated 1-HMP to a mutagen, whereas pulmonary preparations showed much weaker activity (Figure 3 and Table 2). This situation greatly differs from previous findings with rat tissue preparations.[28] Among rat tissues, the liver dominated the activation. Cytosolic preparations from 17 extrahepatic tissues, including the colon and kidney, only demonstrated <0.01% to 1.5% of the activation capacity of the hepatic preparations for 1-HMP. On the contrary, some human extrahepatic tissue preparations showed substantial activation of 1-HMP.[28]

Knockout of *Sult1a1* led to drastic decreases in the hepatic and colonic activation of 1-HMP (by 96% and 74%,

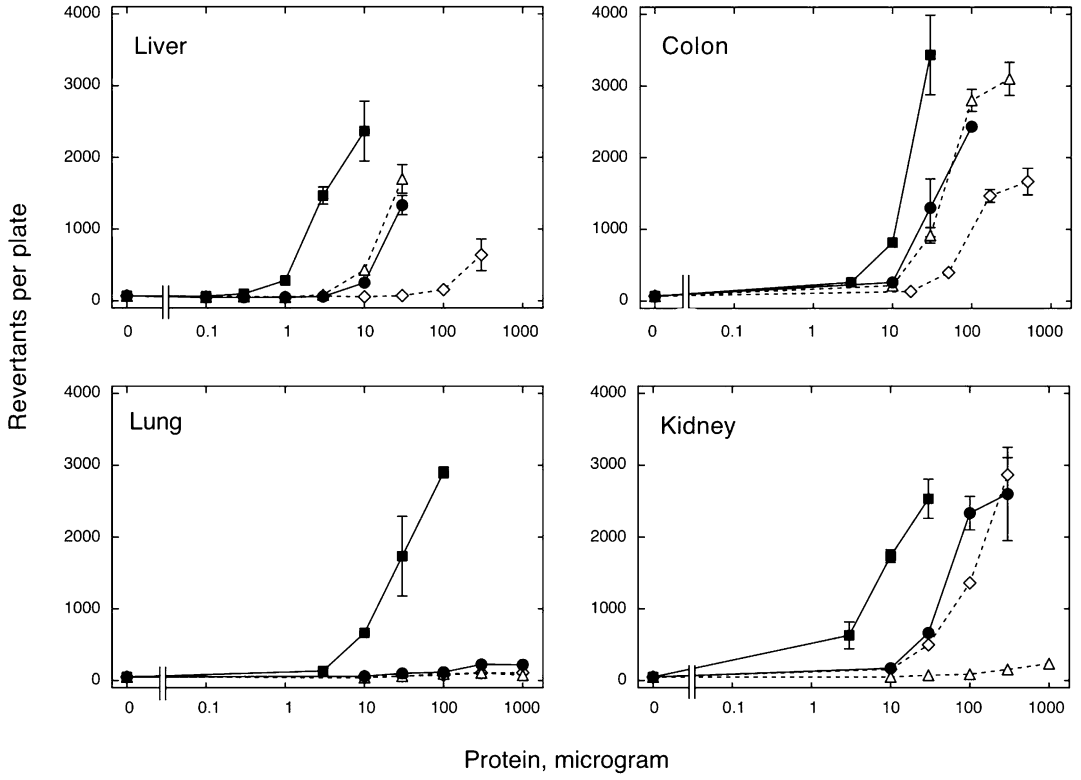

Figure 3. Activation of 1-HMP to a mutagen in *S. typhimurium* TA98 by cytosolic tissue fractions from mouse lines differing in their genetic SULT1A status. Cytosolic fractions were prepared from the liver, colon, lung, and kidney (indicated in the panels) of male mice of the following lines: wild-type (●), knockout of Sult1a1 (◊), knockout of Sult1d1 (△), and transgenic for human SULT1A1/2 (■). Varying amounts of cytosolic fraction (expressed as μg total protein in the abscissa) and the cofactor PAPS (50 μM) were used. Values are the mean ± SE of 3 plates (1-HMP-treated groups) or 3−6 plates (solvent control).

Table 2. Influence of the SULT Status on the Activation of 1-HMP to a Mutagen by Cytosolic Fractions of Various Mouse Tissues[a]

	revertants per μg protein			
SULT genotype	liver	colon	lung	kidney
wild-type	40	30	0.6	23
knockout of Sult1a1	1.5	8	0.2	15
knockout of Sult1d1	50	28	0.4	0.3
transgenic for human SULT1A1/2	460	110	60	190

[a]Mutagenicity was determined in strain TA98 using fixed concentrations of 1-HMP (50 nmol in an incubation volume of 610 μL, i.e., 82 μM) and PAPS (50 μM) and varying amounts of the cytosolic fraction of the indicated sources. Protein−response curves are presented in Figure 3. Values represent the initial slope of these curves.

respectively) compared to that of the wt (Figure 3 and Table 2). Pulmonary activation also appeared to be decreased (but starting from a low level), whereas renal activation was the least affected. This fits reports indicating high mSult1a1 mRNA expression in the liver, colon, and lung but low expression of this form in the kidney.[31] However, the other knockout model used, for Sult1d1, led to a radical decrease in renal activation (by 99%) but hardly affected the activation by the preparations from the other three tissues studied (Figure 3 and Table 2). Indeed, the kidney is the tissue showing the highest expression of *mSult1d1* studied at the mRNA level.[31]

Transgenic hSULT1A1/2 rigorously enhanced the activation of 1-HMP by subcellular preparations from all four tissues studied compared to that in the wt (Figure 3 and Table 2). In absolute terms (expressed as revertants per μg protein), the increase was strongest in the liver. In relative terms (compared to the activation by wt preparations), it was strongest for the lung (100-fold), starting from a low level. Indeed, transgenic hSULT1A1/2 is highly expressed in all of these tissues.[36]

1-SMP Plasma Levels. In the first experiment, wt and hSULT1A1/2-tg mice were intraperitoneally treated with 1-HMP. Plasma 1-SMP levels were determined at various time points. Maximum 1-SMP levels in wt mice were observed at the first analysis time, 15 min after 1-HMP injection (Table 3). Levels then declined rapidly, implying fast metabolism of 1-HMP. Since we hypothesized that 1-HMP may be metabolized even faster in hSULT1A1/2-tg mice, we included an earlier time point, 7.5 min after treatment. Near maximal levels were already attained at this time. The maximal 1-SMP plasma levels, reached after 15 min, outmatched those in the wt by a factor of 9.4. Moreover, the high levels were maintained for a longer period than in the wt. Thus, the ratio of the levels between hSULT1A1/2-tg and wt mice achieved a value of 17.5 after 60 min. The reasons have not been identified. hSULT1A1 might have a lower K_m for 1-HMP than the endogenous SULTs of mouse liver, allowing activation at lower substrate concentrations and, therefore, for an extended period of time. The renal clearance, primarily mediated by organic anion transporters,[24,48] may be saturated at high 1-SMP plasma levels.

In the second experiment, wt, Sult1a1$^-$, and Sult1d1$^-$ mice were treated with 1-HMP following the same regimen as in the first experiment, but analysis of 1-SMP plasma levels was confined to a single time, 30 min after the treatment. Levels in wt animals corresponded to those observed in the initial

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Table 3. Influence of the SULT Status on the Plasma Level of 1-SMP in Mice Treated with 1-HMP[a]

SULT genotype	plasma concentration of 1-SMP (nM)				
	7.5 min	15 min	30 min	60 min	120 min
		Experiment 1			
wild-type	–	510 ± 170	250 ± 110	78 ± 18	28 ± 2
transgenic for human SULT1A1/2	4030 ± 710	4770 ± 1210**	3170 ± 700**	1370 ± 170****	–
		Experiment 2			
wild-type	–	–	270 ± 40	–	–
knockout of Sult1a1	–	–	9 ± 1****	–	–
knockout of Sult1d1	–	–	36 ± 4***	–	–

[a]Adult male mice were killed 7.5−120 min after intraperitoneal treatment with 1-HMP (19.3 mg per kg body mass). The plasma level of 1-SMP was determined using UPLC-MS/MS. Values are the mean ± SE of 5 mice. No 1-SMP or any material that could be confounded with 1-SMP was detected in control animals only treated with the vehicle. Results were statistically analyzed using Student's *t* test: ** $0.01 > p \geq 0.001$; *** $0.001 > p \geq 0.0001$; **** $p < 0.0001$ compared to the wild-type (same time as that of the corresponding experiment). –, not studied.

Table 4. Influence of the Expression of Transgenic Human *SULT1A1/2* on the Formation of DNA Adducts (MPdG) by 1-HMP in Various Mouse Tissues *in Vivo*[a]

tissue	SULT genotype	MPdG adducts per 10^8 dN				
		7.5 min	15 min	30 min	60 min	120 min
liver	wt	–	1330 ± 220	530 ± 70	139 ± 19	134 ± 13
	tg	5000 ± 660	4650 ± 1150*	7120 ± 1420**	1700 ± 310**	–
colon	wt	–	52 ± 9	29 ± 5	<LOD	<LOD
	tg	109 ± 12	64 ± 7 ns	31 ± 5 ns	<LOD	–
lung	wt	–	113 ± 15	120 ± 28	61 ± 6	84 ± 22
	tg	700 ± 126	1350 ± 290**	1000 ± 90**	207 ± 20***	–
kidney	wt	–	143 ± 19	147 ± 28	138 ± 20	116 ± 28
	tg	4040 ± 870	8340 ± 1210***	12200 ± 300****	7200 ± 720****	–
heart	wt	–	<LOD	<LOD	<LOD	<LOD
	tg	159 ± 15	53 ± 1****	43 ± 3****	41 ± 4****	–

[a]Adult male mice, wild-type (wt) or transgenic for human SULT1A1/2 (tg), were killed 7.5−120 min after intraperitoneal treatment with 1-HMP (19.3 mg per kg body mass). The plasma level of 1-SMP was determined using UPLC-MS/MS (liver, kidney, and lung) or ^{32}P-postlabeling (colon and heart). No MPdG or any material that could be confounded with MPdG was detected in the DNA of control animals only treated with the vehicle. Values are the mean ± SE of 5 mice. Results were statistically analyzed using Student's *t* test: n.s., not significant ($p \geq 0.05$); * $0.05 > p \geq 0.01$; ** $0.01 > p \geq 0.001$; *** $0.001 > p \geq 0.0001$; **** $p < 0.0001$ compared to the wild-type (same treatment time). –, not studied; LOD, limit of detection (10 adducts per 10^8 dN).

experiment. Levels in Sult1a1$^-$ and Sult1d1$^-$ mice were reduced compared to those in the wt by 97% and 87%, respectively, inferring that Sult1a1 as well as Sult1d1 were involved in the sulfation of 1-HMP *in vivo*. It was somewhat surprising that the sum of these reductions was greater than 100%. We speculate that a small decrease in intracellular 1-SMP may decrease plasma 1-SMP levels overproportionally, e.g., due to improved detoxification or renal clearance.

DNA Adducts in Mouse Models. DNA adducts were determined in the same mice as those used for the analysis of 1-SMP plasma levels. In wt mice, the highest adduct levels were formed in the liver, followed, with much distance, by the kidney, lung, and colon; no adducts were detected in the heart (Tables 4 and 5). In the liver, maximal adduct levels were reached extraordinarily quickly (15 min after treatment), followed by a rather speedy decline: a decrease by 90% in the period from 15 to 120 min after the treatment (Table 4). Since the MPdG adducts are chemically stable,[21] this finding suggests a high rate of repair, at least in the initial phase. Indeed, 1-HMP is a very potent inducer of unscheduled DNA synthesis in SULT-expressing cell lines.[49] Time-dependent decreases in the adduct levels in wt mice were also observed in the lung and colon, but not in the kidney. We suspect that repaired renal adducts were outbalanced by adducts newly generated from 1-SMP taken up into proximal tubule cells from the circulation, as outlined previously.[24,48]

Table 5. Influence of Sult1a1 and Sult1d1 Knockout on the Formation of DNA Adducts by 1-HMP in Various Mouse Tissues *in Vivo*[a]

SULT genotype	MPdG adducts per 10^8 dN			
	liver	colon	lung	kidney
wild-type	522 ± 72	29 ± 11	110 ± 27	122 ± 23
knockout of Sult1a1	55 ± 10***	39 ± 4 ns	50 ± 10 ns	43 ± 8*
knockout of Sult1d1	292 ± 46*	67 ± 14*	64 ± 8 ns	64 ± 8*

[a]Adult male mice were killed 30 min after oral treatment with 1-HMP (19.3 mg per kg body mass). The level of MPdG adducts was determined using the isotope-dilution UPLC-MS/MS method. No MPdG or any material that could be confounded with MPdG was detected in the DNA of control animals only treated with the vehicle. Values are the mean ± SE of 5 mice. Results were statistically analyzed using Student's *t* test: n.s., not significant; ($p \geq 0.05$); * $0.05 > p \geq 0.01$; *** $0.001 > p \geq 0.0001$ compared to the wild-type.

The time courses of the DNA adduct level were also determined in hSULT1A1/2-tg mice (Table 4). The tissue distribution of the adducts was altered in these mice compared to that in the wt due to tissue-selective increases in the adduct

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levels. The increases in the peak adduct levels were 83-fold for the kidney, 11-fold for the lung, 5.4-fold for the liver, and 2.1-fold (statistically not significant) for the colon, resulting in the following order of peak adduct levels: kidney > liver > lung > > colon. In addition, DNA adducts were detected in the heart tissue of hSULT1A1/2-tg mice but not of wt mice.

Hepatic and renal DNA adduct levels in Sult1a1$^-$ mice were diminished by 89% and 65%, respectively (Table 5). Both influences were statistically significant. In addition, pulmonary adducts were decreased by 45%, and colonic adducts were increased by 34% in Sult1a1$^-$ mice, but both changes were not statistically corroborated. The decrease in the hepatic adduct level is plausible, inasmuch as the liver tissue is rich in Sult1a1, a form that efficiently activates 1-HMP. In contrast, the kidney does not express appreciable levels of this form.[31] Thus, it is likely that the renal effect of Sult1a1 is due to the drastic decrease in the 1-SMP level in blood plasma (Table 3) and, therefore, reduced renal uptake from this source.

Sult1d1 knockout had milder impacts on the adduct levels than Sult1a1 knockout. Renal adduct levels were reduced in Sult1d1$^-$ mice by 48% (Table 5). This finding can be rationalized by the high expression of *Sult1d1* in the kidney of wt mice.[31] In addition, colonic adduct levels were increased 2.3-fold ($p < 0.05$) in Sult1d1$^-$ mice. This effect is not simple to understand. Perhaps, more 1-HMP reaches the colon of Sult1d1$^-$ mice, as compared to that of wt mice, and is activated in this tissue by other SULT forms, such as Sult1a1. Moreover, the metabolism of 1-HMP may be somewhat delayed in Sult1d1$^-$ mice, compared to that in the wt, producing a temporal shift of the maximum adduct level (a factor studied in SULT1A1/2-tg versus wt mice but not in the knockout lines).

CONCLUSIONS

In the present study, we demonstrate that several mouse SULT forms are able to toxify 1-HMP. Moreover, various mouse tissues, including the liver, colon, and kidney, comprise high activity of this reaction implying a striking difference from the situation found in the rat, in which the activity is rather restricted to the liver. Species-dependent differences were also observed in the tissue distribution of the DNA adducts formed in 1-HMP-treated animals. In wt mice, the highest adduct levels occurred in the liver. In the rat, the highest level were found in the kidney, 3-fold above the hepatic level,[24] despite the low renal 1-HMP sulfoconjugation activity.[28] In both species, the order of the adduct levels in the liver and kidney could be inverted by some manipulations. When rats were cotreated with 1-HMP and probenecid, an inhibitor of organic anion transporters (mainly located in the proximal tubule cells) and other trans-membrane transporters (e.g., located in the sinusoidal and apical membranes of the hepatocytes), hepatic MPdG adduct levels were enhanced 23-fold, whereas those in the kidneys were slightly decreased.[24] As a result, hepatic adduct levels exceeded the renal levels by a factor of 12. This paradigm demonstrates that transport processes play pivotal roles in the tissue distribution of adducts formed by 1-HMP. In the mouse, the highest adduct formation was shifted from the liver to the kidney by the expression of human *SULT1A1/2*. The 83-fold increase in the renal adduct level, compared to that of wt animals (calculated from the peak levels), was associated with a 9.4-fold increase in the plasma 1-SMP concentration. Therefore, it is probable that 1-SMP produced in the liver and other extrarenal tissues substantially contributed to the enhanced renal adduct formation in SULT1A1/2-tg mice. However, we suspect that there was also an input of local activation, as the transgene enhanced the renal activation capacity 8-fold (Table 2). Likewise, knockout of Sult1d1, a form preferentially expressed in the kidney, reduced renal 1-HMP activation *in vitro* by 99% (Table 2) and renal adduct formation *in vivo* by 48% (Table 5).

The colon showed high 1-HMP activation capacity *in vitro* (e.g., 75% of the hepatic capacity in wt mice; Table 2), but DNA adduct formation in this tissue remained low (nearly 5% compared to that in the liver in wt mice; Tables 4 and 5). The low colonic adduct formation may be because of the very fast metabolism of 1-HMP and the relatively low perfusion rate of the colon compared to that of the liver, lung, and kidney.

Although several mouse SULT forms were able to activate 1-HMP (Table 1), knockout of a single form, Sult1a1, demonstrated dramatic effects on DNA adduct formation in the liver (decrease by 89%, Table 5) and on plasma 1-SMP concentration (decrease by 97%, Table 3). Such a dominant impact of Sult1a1 was also observed with other genotoxicants. Thus, hepatic DNA adduct formation by methyleugenol and 1′-hydroxymethyleugenol was reduced by up to 97% and 99.2%, respectively, in Sult1a1$^-$ mice compared to those in the wt.[37] Likewise, Sult1a1 knockout drastically reduced the hepatic DNA adduct formation by 1-methoxyindole-3-carbinol (Weißenberg, S., Meinl, W., Schumacher, F., Lampen, A., Ehlers, A., and Glatt, H. R., manuscript in preparation), another SULT-dependent genotoxicant.[50,51] The finding of a dominating role of mSult1a1 with various genotoxicants is important for risk assessment, as the corresponding human enzyme, SULT1A1, shows common single-nucleotide and copy-number polymorphisms.[33,52−54]

However, this finding cannot be generalized to all SULT-dependent gentoxicants. We have detected a substantial number of genotoxicants that are activated by certain SULT forms but not by hSULT1A1 or mSult1a1.[26,27,29]

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ABBREVIATIONS

dN, 2′-deoxynucleoside(s); ESI+, positive electrospray ionization; 1-HMP, 1-hydroxymethylpyrene; LOD, limit of detection;

MPdG, N^2-(1-methylpyrenyl)-2′-deoxyguanosine; MRM, multiple reaction monitoring; MS, mass spectrometry; PAH, polycyclic aromatic hydrocarbon(s); PAPS, 3′-phosphoadenosine-5′-phosphosulfate; 1-SMP, 1-sulfooxymethylpyrene; SULT, sulfotransferase (with prefix h for human and m for mouse, when not clear from the context); SULT1A1/2-tg, hemizygously transgenic for human SULT1A1/2; UPLC, ultraperformance liquid chromatography; wt, wild-type

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