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Sulphation of acetaminophen by the human cytosolic sulfotransferases: a systematic analysis

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Sulphation is known to be critically involved in the metabolism of acetaminophen *in vivo*. This study aimed to systematically identify the major human cytosolic sulfotransferase (SULT) enzyme(s) responsible for the sulphation of acetaminophen. A systematic analysis showed that three of the twelve human SULTs, SULT1A1, SULT1A3 and SULT1C4, displayed the strongest sulphating activity towards acetaminophen. The pH dependence of the sulphation of acetaminophen by each of these three SULTs was examined. Kinetic parameters of these three SULTs in catalysing acetaminophen sulphation were determined. Moreover, sulphation of acetaminophen was shown to occur in HepG2 human hepatoma cells and Caco-2 human intestinal epithelial cells under the metabolic setting. Of the four human organ samples tested, liver and intestine cytosols displayed considerably higher acetaminophen-sulphating activity than those of lung and kidney. Collectively, these results provided useful information concerning the biochemical basis underlying the metabolism of acetaminophen *in vivo* previously reported.

Keywords: acetaminophen/cytosolic sulfotransferase/sulfation/SULT.

Abbreviations: ATP, adenosine 5′-triphosphate; CAPS, 3-(cyclohexylamino)-1-propanesulphonic acid; CHES, 2-(cyclohexylamino)ethanesulphonic acid; DTT, dithiothreitol; FBS, fetal bovine serum; HEPES, N- 2-hydroxylpiperazine-N′-2-ethanesulphonic acid; MEM, minimum essential medium; MES, morpholinoethanesulphonic acid; PAPS, 3′-phosphoadenosine 5′-phosphosulphate; SULT, cytosolic sulfotransferase; TLC, thin-layer chromatography.

Acetaminophen (*N*-(4-hydroxyphenyl)acetamide) is one of the most widely used drugs for treating pain and fever. When used at recommended therapeutic doses, acetaminophen is relatively safe (*1*). A range of adverse effects including hepatotoxicity (*2*, *3*), however, have been reported with acetaminophen overdose. When taken orally, acetaminophen is readily absorbed from the gastrointestinal tract with concentrations peaking in plasma within 10–60 min (*4*, *5*). The elimination half-life of acetaminophen has been determined to be between 1 and 3 h (*4*, *6*). The metabolism of acetaminophen has been reported to take place predominantly in the liver and excreted in the urine mainly as glucuronidated and sulphated metabolites (*7–10*). A recent report showed that 25–35% of a therapeutic dose of acetaminophen was recovered in sulphated form (*11*).

In humans and other mammals, sulphate conjugation as catalysed by the cytosolic sulfotransferases (SULTs) is known to be involved in the biotransformation of a variety of xenobiotics including many drugs (*12–14*). In SULT-mediated reactions, a sulphonate group is transferred from the active sulphate, 3′-phosphoadenosine 5′-phosphosulphate (PAPS), to a hydroxyl or amino group-containing substrate compound (*15*). Sulphate conjugation may result in the inactivation of the substrate compounds and/or their disposal from the body (*16*). In humans, there are thirteen SULTs that are classified into four distinct gene families, designated as SULT1, SULT2, SULT4 and SULT6 (*17–19*). Several of them, including SULT1A1 and SULT1A3, have been sporadically reported to be capable of sulphating acetaminophen (*20–22*). To better understand the involvement of SULT-mediated sulphation in the metabolism of acetaminophen, it is important to obtain more detailed information concerning the identities and enzymatic characteristics of the SULTs that are capable of sulphating acetaminophen.

We report in this communication a systematic investigation of the sulphating activity of the human SULTs towards acetaminophen. The pH dependence and kinetic parameters of the major acetaminophen-sulphating SULTs were determined. Moreover, the sulphation of acetaminophen under metabolic conditions and by human organ specimens was examined.

Materials and Methods

Materials

Acetaminophen (*N*-(4-hydroxyphenyl)acetamide) was a product of Cerilliant Corporation (Round Rock, TX). Adenosine 5′-triphosphate (ATP), PAPS, 2-morpholinoethanesulphonic acid

(MES), 3-(*N*-morpholino)propanesulphonic acid (MOPS), *N*-2-hydroxylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), 3-[*N*-tris-(hydroxymethyl)methylamino]-propanesulphonic acid (TAPS), 2-(cyclohexylamino)ethanesulphonic acid (CHES), 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS), dithiothreitol (DTT) and minimum essential medium (MEM) were products of Sigma Chemical Company (St. Louis, MO,). Fetal bovine serum (FBS) was from Biomeda (Foster City, CA). HepG2 human hepatoma cell line (ATCC HB-8065) and Caco-2 human intestinal epithelial cell line (ATCC HTB-37) were obtained from American Type Culture Collection (Manassas, VA). Cytosols of human intestine, kidney liver and lung were products of XenoTech, LLC (Lenexa, KS). Cellulose thin-layer chromatography (TLC) plates were from EMD Millipore Corporation (Billerica, MA). Ecolume scintillation cocktail was purchased from MP Biomedicals, LLC. (Irvine, CA). All other chemicals were of the highest grade commercially available.

Preparation of purified human SULTs

Recombinant P-form (SULT1A1 and SULT1A2) and M-form (SULT1A3) phenol SULTs, the thyroid hormone SULT (SULT1B1), three SULT1Cs (SULT1C2, SULT1C3 and SULT1C4), the oestrogen SULT (SULT1E1), the dehydroepiandrosterone (DHEA) SULT (SULT2A1), two SULT2B1s (SULT2B1a and SULT2B1b), a neuronal SULT (SULT4A1) and a SULT6B1, expressed using pGEX-2TK or pET23c prokaryotic expression system, were prepared as described previously (*23*–*27*).

SULT assay

The sulphating activity of the recombinant human SULTs was assayed using PAP[^{35}S] as the sulphonate group donor. The standard assay mixture, in a final volume of 20 µl, contained 50 mM of HEPES buffer at pH 7.0, 1 mM DTT and 14 µM PAP[^{35}S]. Stock solution of acetaminophen, dissolved in dimethyl sulphoxide, was used in the enzymatic assay. Acetaminophen, at 10 times the final concentration (50 µM) in the assay mixture, was added after HEPES buffer and PAP[^{35}S]. The reaction was started by the addition of the SULT enzyme, allowed to proceed for 5 min at 37°C and terminated by placing the thin-walled tube containing the assay mixture on a heating block, pre-heated to 100°C, for 3 min. The precipitates were cleared by centrifugation at 15,000 × *g* for 3 min, and the supernatant was subjected to the analysis of [^{35}S] sulphated product using the TLC procedure with n-butanol:isopropanol:88% formic acid:water (3:1:1:1; by volume) as the solvent system (*28*). Upon completion of TLC, the TLC plate was air dried and autoradiographed by using an X-ray film (Thermo Fisher Scientific Inc., MA). The radioactive spot corresponding to sulphated acetaminophen was located, cut out and eluted in 0.5 ml water in a shell vial. Afterwards, 4.5 ml of the Ecolume scintillation liquid was added to each vial, mixed thoroughly and the radioactivity therein was counted using a liquid scintillation counter. To assay for acetaminophen-sulphating activity of human organ cytosols, the reaction mixture was supplemented with 50 mM NaF (a phosphatase inhibitor). The reaction was started by the addition of the cytosol and allowed to proceed for 20 min, followed by the TLC analysis for [^{35}S]sulphated product as described above. To examine the pH dependence of the sulphation of acetaminophen by human SULT1A1, SULT1A3 and SULT1C4, different buffers (sodium acetate at 4.5, 5.0 or 5.5; MES at 5.5, 6.0 or 6.5; MOPS at 6.5, 7.0 or 7.5; HEPES at 7.0, 7.5 or 8.0; TAPS at 8.0 or 8.5, 9.0; CHES at 9.0, 9.5 or 10.0; and CAPS at 10.0, 10.5, 11.0 or 11.5) at 50 mM concentration, instead of 50 mM HEPES (pH 7.0), were used in individual reactions. Each experiment was performed in triplicate, together with a control without substrate. The results obtained were calculated and expressed in nanomoles or picomoles of sulphated product formed/min/mg purified enzyme. In the kinetic studies on the sulphation of acetaminophen, the SULT assays were carried out using varying concentrations (ranging 0–4,000 µM) of acetaminophen and 50 mM HEPES at pH 7.0 according to the procedure described above. Data obtained were analysed based on Michaelis–Menten kinetics using KaleidaGraph 4.1 software (Synergy Software Inc., PA) and non-linear regression.

Metabolic labelling of HepG2 human hepatoma cells and Caco-2 human intestinal epithelial cells

HepG2 cells and Caco-2 cells were maintained, under a 5% CO_2 atmosphere at 37°C, in MEM supplemented with 10% FBS, penicillin G (30 µg/ml) and streptomycin sulphate (50 µg/ml). Confluent cells grown in individual wells of a 24-well culture plate, pre-incubated in sulphate-free (prepared by omitting streptomycin sulphate and replacing magnesium sulphate with magnesium chloride) MEM with 10% dialysed FBS for 4 h, were labelled with 0.25 ml aliquots of the same medium containing [^{35}S]sulphate (0.3 mCi/ml) plus different concentrations (0, 5, 10, 25, 50 and 100 µM) of acetaminophen. At the end of an 18-h labelling period, the media were collected, spin-filtered to remove high-molecular weight [^{35}S]sulphated macromolecules, and subjected to thin-layer analysis for [^{35}S]sulphated acetaminophen based on the procedure described above.

Miscellaneous methods

PAP[^{35}S] was synthesized from ATP and carrier-free [^{35}S]sulphate using the bifunctional human ATP sulfurylase/adenosine 5′-phosphosulphate kinase (*29*). PAP[^{35}S] synthesized was adjusted to the required concentration and a specific activity of 15 Ci/mmol at 1.4 mM by the addition of non-radioactive PAPS. Protein determination was based on the method of Bradford with bovine serum albumin as the standard (*30*).

Results and Discussion

Previous studies indicated that sulphation constitutes a major pathway in the metabolism of acetaminophen *in vivo* (*31*, *32*). While sporadic studies had revealed acetaminophen-sulphating activity of several human SULTs, a complete account of those responsible enzymes is lacking. This study was designed to systematically identify the human SULTs that are capable of sulphating acetaminophen and to examine their enzymatic characteristics in mediating acetaminophen sulphation. Moreover, sulphation of acetaminophen under the metabolic setting was investigated using HepG2 human hepatoma cells and Caco-2 human intestinal epithelial cells. Acetaminophen-sulphating activity of cytosols prepared from four human organs, liver, intestine, lung and kidney, was evaluated.

Differential sulphating activities of the human SULTs towards acetaminophen

To identify the enzymes that are capable of sulphating acetaminophen, the 13 human SULTs, previously cloned, expressed and purified, were examined for sulphating activity with 50 µM of acetaminophen as a substrate. Results obtained showed that 10 (SULT1A2, SULT1B1, SULT1C2, SULT1C3, SULT1E1, SULT2A1, SULT2B1a, SULT2B1b, SULT4A1 and SULT6B1) of the 13 SULTs showed no detectable activities. It should be pointed out that a previous study showed acetaminophen-sulphating activity for SULT1E1 and SULT2A1 (*22*). It is noted, however, that a substrate concentration of 16 mM of acetaminophen was tested in that study, versus 50 µM of acetaminophen used in this study. The much lower substrate concentration used in this study is likely the reason why no acetaminophen-sulphating activity was detected for SULT1E1 and SULT2A1. Of the other three SULTs (SULT1A1, SULT1A3 and SULT1C4), SULT1C4 exhibited the strongest acetaminophen-sulphating activity, followed by SULT1A1 and SULT1A3 (Table I). It is noted that SULT1A1 and SULT1A3 are the two 'phenol SULTs' that were first identified back in the early 1980s

Table I. Specific activities of human SULT1A1, SULT1A3 and SULT1C4 with acetaminophen, *p*-nitrophenol and 2-naphthol as substrates[a]

	Specific activity (nmol/min/mg)		
Substrate	**SULT1A1**	**SULT1A3**	**SULT1C4**
Acetaminophen	11.52 ± 0.51	5.20 ± 0.36	30.14 ± 1.16
p-Nitrophenol	21.98 ± 1.04	2.59 ± 0.11	43.00 ± 0.70
2-Naphthol	23.90 ± 0.24	7.66 ± 0.19	43.82 ± 0.62

[a]Specific activity refers to nmol substrate sulphated /min/mg purified enzyme. Data represent mean ± SD derived from three determinations.

(*20*, *33*). These two enzymes displayed substrate specificity towards compounds containing phenolic (aryl) hydroxyl groups in their chemical structures (*20*, *33*; cf. Table I). It is therefore not surprising that acetaminophen, which contains a phenolic hydroxyl group, serves as a good substrate for these two enzymes. For SULT1C4, although its crystal structure is still not available, it may also have an active site structure that favours the binding of substrates carrying phenolic hydroxyl groups. In regard to the tissue specificity of expression, previous studies have demonstrated that SULT1C4 is expressed in several human organs including the fetal kidney and lung, as well as adult ovary and kidney (*24*). SULT1A1 has been shown to be expressed in the liver, as well as brain, gastrointestinal tract, platelets and placenta (*34*). SULT1A3, on the other hand, is expressed at high levels in the jejunum, gastrointestinal tract, platelets, brain and fetal liver (*35*). These three SULT enzymes, expressed in different organs and at different developmental stages, therefore may act to metabolize acetaminophen through sulphation.

Characterization of the acetaminophen-sulphating activity of human SULT1A1, SULT1A3 and SULT1C4

To investigate further the sulphation of acetaminophen by the human SULTs, the pH dependence and kinetic parameters of SULT1A1, SULT1A3 or SULT1C4-mediated sulphation of acetaminophen were analysed. As shown in Fig. 1, the pH-dependence of the sulphation of acetaminophen differed considerably among the three human SULTs. SULT1A1 showed a high level of sulphating activity spanning pH 6.5–8 (Fig. 1A), whereas SULT1A3 displayed a distinct optimum at pH 9.5 (Fig. 1B). SULT1C4, on the other hand, showed a pH optimum spanning 7–8.5 (Fig. 1C). The differential pH-dependence profiles of these three SULTs may reflect their distinct substrate-binding and/or catalytic residues involved in mediating the sulphation of acetaminophen. Figure 2 shows the amino acid sequence alignment of SULT1A1, SULT1A3 and SULT1C4. As indicated, all these three SULTs contain the so-called 'signature sequences' involved in the binding of the co-substrate, PAPS (*36*). Moreover, two residues, lysine 48 and histidine 108, previously shown to be critical for the catalytic activity of both SULT1A1 and SULT1A3 (*37*), are also conserved in SULT1C4. Examining the

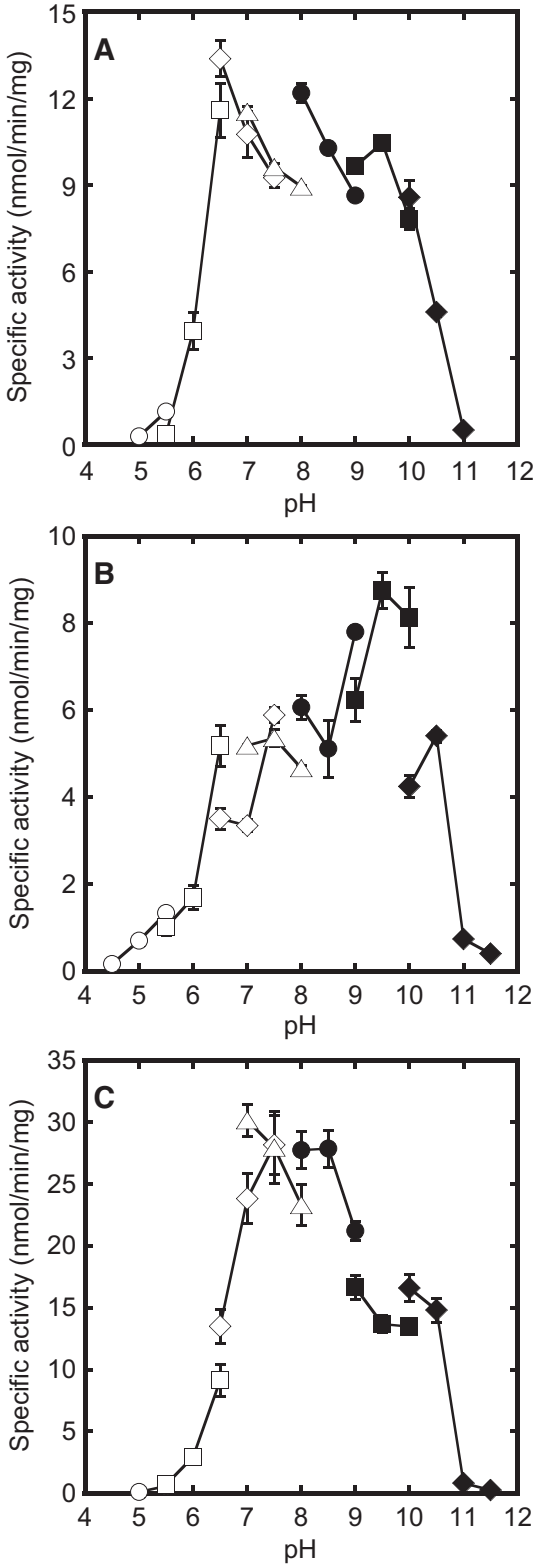

Fig. 1 pH dependency of acetaminophen-sulphating activity of the human SULT1A1 (A), SULT1A3 (B) and SULT1C4 (C). Enzymatic assays were carried out under standard assay conditions as described in the Materials and Methods section using different buffer systems as indicated. Data shown represent calculated mean ± SD derived from three independent experiments. Symbols used are open circle for the acetate buffer at pH 4.5–5.5; open square for MES buffer at pH 5.5–6.5; open diamond for MOPS buffer at pH 6.5–7.5; open triangle for HEPES buffer at pH 7.0–8.0; closed circle for TAPS buffer at pH 8.0–9.0; closed square for CHES buffer at pH 9.0–10.0 and closed diamond for CAPS buffer at pH 10.0–11.5.

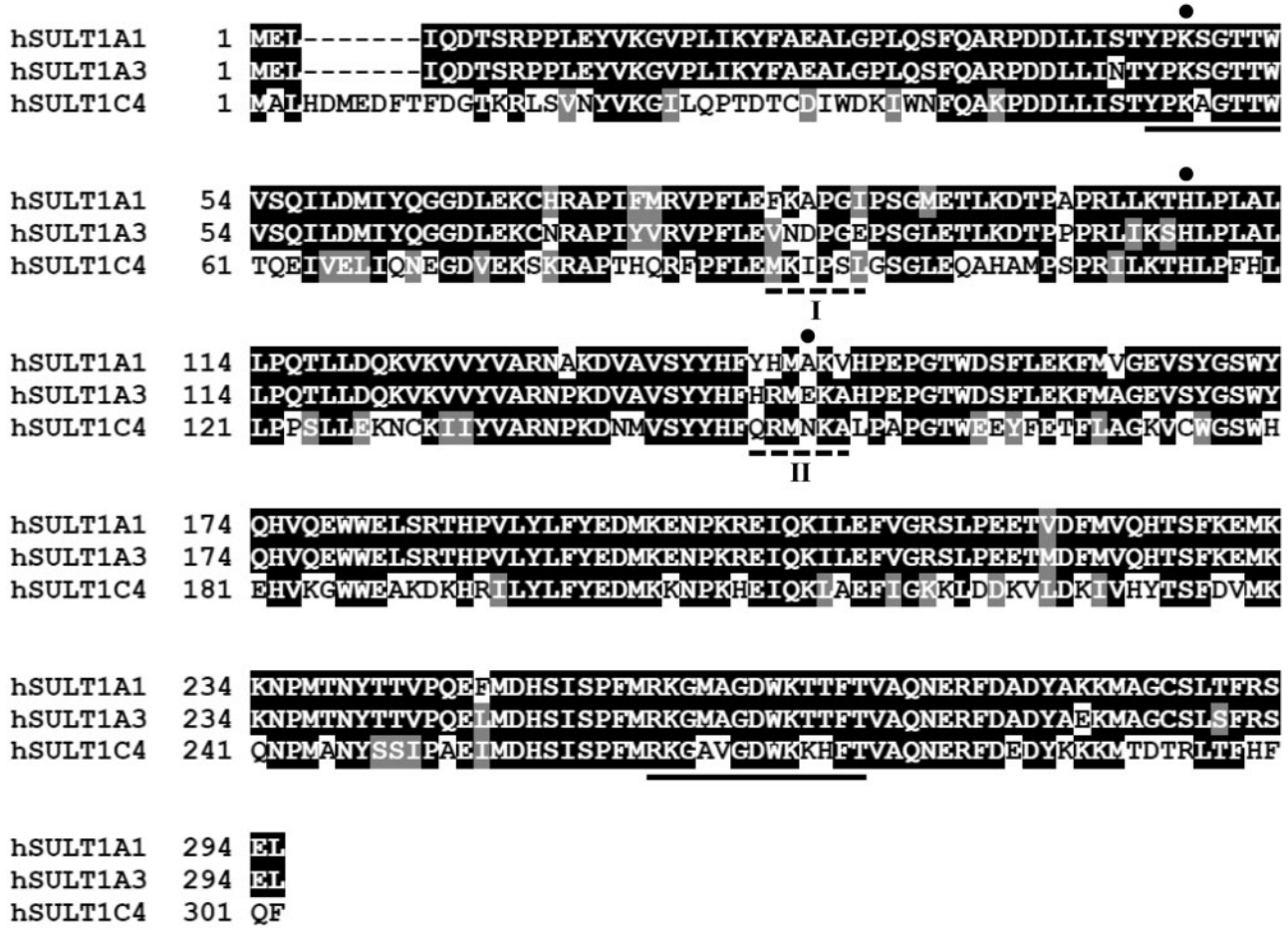

Fig. 2 Amino acid sequence alignment of human SULT1A1, SULT1A3 and SULT1C4. Identical amino acid residues are marked by black background and similar amino acid residues are marked by grey background. Solid lines indicate the 'signature sequences' involved in the binding of PAPS. Dashed lines indicate the variable regions I and II previously identified for SULT1A1 and SULT1A3. Round solid spots indicate Lys48 and His108, previously shown to be critical to the catalytic activity of SULT1A1 and SULT1A3.

'variable regions' I and II, previously characterized to be important for the substrate specificity of SULT1A1 and SULT1A3 (*23*), it is noted that SULT1C4 shows both similarities and differences to SULT1A1 and SULT1A3 in both variable regions. While it remains to be clarified, it is possible that some of the amino acid residues, which carry ionizable side chains with different pKa values, in these two regions may be involved in substrate-binding and therefore the differential pH optima of these three SULT enzymes. The kinetics of the sulphation of acetaminophen by SULT1A1, SULT1A3 and SULT1C4 were subsequently analysed using varying concentrations of acetaminophen as substrates. As shown in Fig. 3, the sulphation of acetaminophen appeared to follow the Michaelis–Menten kinetics. Table II summarizes the kinetic constants determined for each of the three SULTs in catalysing the sulphation of acetaminophen. SULT1C4 showed the lowest K_m (172.5 μM) which is more than two times lower than those of SULT1A1 (407.9 μM) and SULT1A3 (634.6 μM), indicating that the affinity of SULT1C4 for acetaminophen is much higher than that of SULT1A1 and SULT1A3. The catalytic efficiency of SULT1C4, as reflected by the k_{cat}/K_m (26.54), was 3.35 and 6.06 times higher than that of SLUT1A1 (7.92) and SULT1A3 (4.38). These results clearly indicated that SULT1C4 is capable of catalysing the sulphation of acetaminophen much more efficiency than SULT1A1 and SULT1A3.

An interesting issue is related to the very weak substrate inhibition (above 3 mM) observed with SULT1A1 (cf. Panel A of Fig. 3). Previous studies have demonstrated that SULT1A1 may be subjected to strong substrate inhibition, *e.g.* at μM levels in the case of *p*-nitrophenol (*38*, *39*). While the molecular basis for the differential substrate inhibition remains to be clarified, structural studies have suggested the involvement of two substrate-binding pockets, of which one is involved in catalysis, of the SULT1A1 molecule (*38*, *39*). It has been proposed that the binding of a substrate, such as *p*-nitrophenol, into the catalytic pocket may alter the conformation of the second substrate-binding pocket, particularly in regard to the spatial arrangement of Phe276 and Phe84 residues, which in turn may affect the catalytic efficiency (*40*). The spatial arrangement of the second substrate-binding pocket, intriguingly, varies with substrates (*40*). To gain insight into the molecular basis of the weak substrate inhibitory effect of acetaminophen, docking simulation was performed based on two

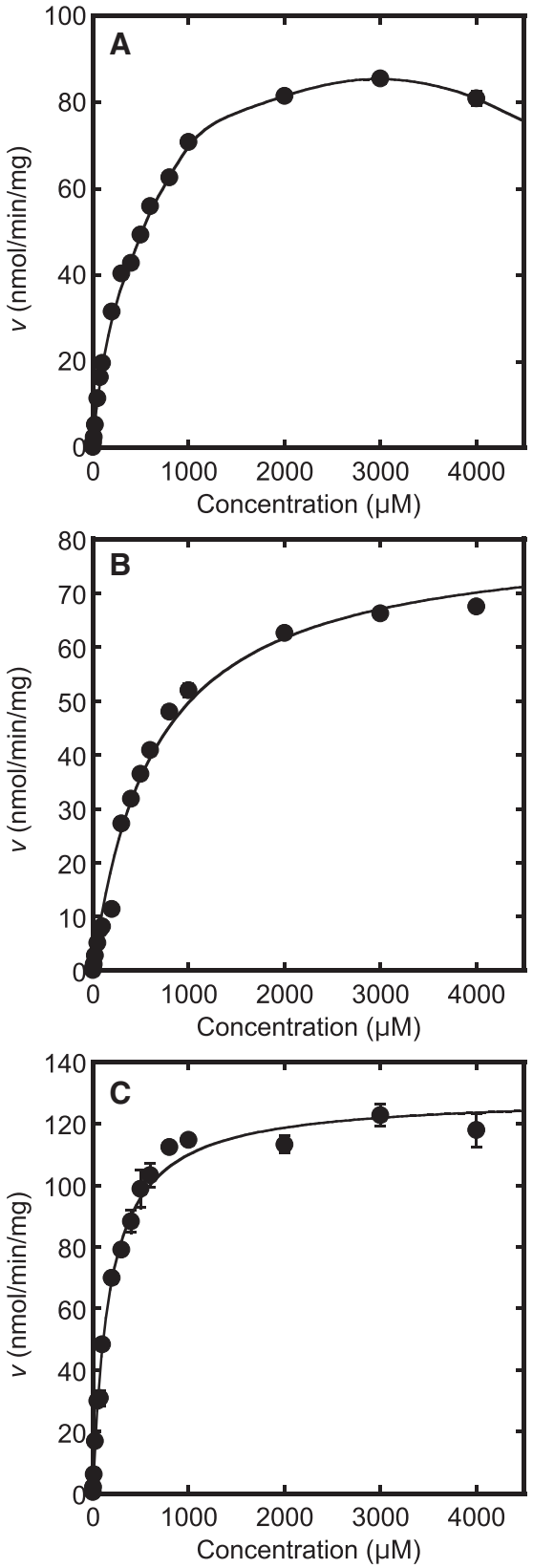

Fig. 3 Kinetic analysis of the sulphation of acetaminophen by human SULT1A1, SULT1A3 and SULT1C4. The figure shows the saturation curve analyses of the sulphation of acetaminophen. The fitting curves were generated based on Michaelis–Menten kinetics. Data shown represent calculated mean ± SD derived from three experiments.

Table II. Kinetic constants of the sulphation of acetaminophen by human SULT1A1, SULT1A3 and SULT1C4[a]

	V_{max} (nmol/min/mg)	k_{cat} (min^{-1})[b]	K_m (μM)	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)
SULT1A1	94.62 ± 0.98	3.23 ± 0.03	407.9 ± 7.67	7.92
SULT1A3	81.21 ± 0.68	2.78 ± 0.02	634.6 ± 4.02	4.38
SULT1C4	128.9 ± 0.19	4.58 ± 0.01	172.5 ± 4.20	26.54

[a]Data shown represent means ± SD derived from three determinations. Kinetic parameters were determined based on Michaelis–Menten kinetics.
[b]k_{cat} values were calculated from the V_{max} values based on the molecular weights of 34,165, 34,196 and 35,520, respectively, for SULT1A1, SULT1A3 and SULT1C4.

different SULT1A1 structures, designated PDB code 1LS6 (*38*) and 3U3K (*40*), using the AutoDock Vina software (*41*). For the 1LS6 structure (complexed with *p*NP), which has an opened space in its second substrate-binding pocket, acetaminophen could be docked into the space between Phe276 and Phe84 (data not shown). In contrast, for the 3U3K structure (complexed with 2-naphthol; known to exhibit minimum substrate inhibitory effect), which takes on a closed space in its second-binding pocket, acetaminophen failed to dock into the space (data not shown). These observations therefore imply that the binding of an acetaminophen molecule to the catalytic pocket, like that with 2-naphthol, may also alter the conformation of SULT1A1 in such a way that would not allow the binding of a second acetaminophen molecule required for exerting the substrate inhibition effect. Further crystal structure studies are warranted in order to fully understand the molecular basis underlying the minimum substrate inhibition of SULT1A1 by acetaminophen as a substrate.

Generation and release of [^{35}S]sulphated acetaminophen by HepG2 cells and Caco-2 cells labelled with [^{35}S]sulphate in the presence of acetaminophen

HepG2 human hepatoma cells and Caco-2 human intestinal epithelial cells were used to investigate whether sulphation of acetaminophen occurs under the metabolic setting. HepG2 human hepatoma cells are known to express several human SULTs including SULT1A1, SULT1A2, SULT1A3, SULT1E1 and SULT2A1 (*42*, *43*). Caco-2 human intestinal epithelial cells are known to express SULT1A1, SULT1A2, SULT1A3, SULT1B1, SULT1C2, SULT1C4 and SULT2A1 (*44*). Confluent HepG2 cells and Caco-2 cells grown in individual wells of a 24-well plate were labelled with [^{35}S]sulphate in sulphate-free medium containing different concentrations of acetaminophen. At the end of an 18-h labelling, the spent labelling medium samples were collected and analysed by TLC. As shown in Fig. 4, a significant amount of [^{35}S]sulphated acetaminophen was detected in spent labelling medium containing as low as 5 μM of acetaminophen, which continued to increase with increasing concentrations of acetaminophen added to the labelling media. These results indicated clearly that

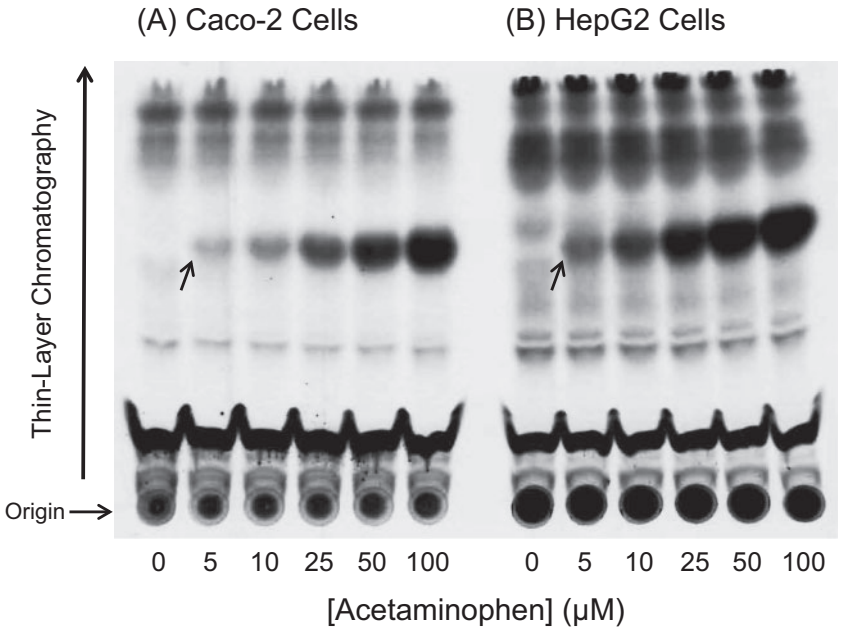

Fig. 4 Analysis of the [^{35}S]sulphated product generated and released by HepG2 human hepatoma cells and Caco-2 human intestinal epithelial cells labelled with [^{35}S]sulphate in the presence of acetaminophen. Confluent HepG2 cells and Caco-2 cells were labelled with [^{35}S]sulphate in the presence of different concentrations (0, 5, 10, 25, 50 and 100 μM) of acetaminophen. At the end of an 18-h labelling, spent labelling media were collected and analysed by TLC. The figure shows the autoradiograph taken from the cellulose plate used for the TLC analysis.

Table III. Sulphating activity of human lung, liver, kidney and intestine cytosols with acetaminophen as a substrate[a]

Specific activity (pmol/min/mg protein)			
Lung	**Liver**	**Kidney**	**Intestine**
3.24 ± 0.39	14.62 ± 0.76	1.77 ± 0.76	20.16 ± 1.37

[a]Specific activity refers to pmol substrate sulphated/min/mg protein. Data represent mean ± SD derived from three determinations. The concentration of the substrate used in the assay mixture was 50 μM.

sulphation of acetaminophen may indeed occur in cells under the metabolic setting.

Sulphation of acetaminophen by human organ samples

To evaluate acetaminophen-sulphating activity *in vivo*, enzymatic assays were performed using cytosols prepared from human lung, liver, kidney or intestine. Activity data obtained are compiled in Table III. Of the four human organ samples tested, cytosols prepared from human intestine and liver exhibited considerably higher acetaminophen-sulphating activity than the cytosols prepared from human lung and kidney. These results indicated that liver and intestine are likely major human organs involved in the metabolism of acetaminophen through sulphation.

Conclusions

This study revealed three human SULTs, SULT1A1, SULT1A3 and SULT1C4, as the major human SULTs capable of sulphating acetaminophen. Metabolic labelling experiments revealed clearly the generation and release of [^{35}S]sulphated acetaminophen by cultured HepG2 human hepatoma cells and Caco-2 human intestinal epithelial cells. Moreover, acetaminophen-sulphating activity was detected, in decreasing order, in human intestine, liver, lung and kidney. These results provided useful information relevant to the biochemical basic underlying the previous findings on the metabolism of acetaminophen *in vitro* and *in vivo*.

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Conflict of Interest
None declared.

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