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Comparison of 2-aminophenol and 4-nitrophenol as *in vitro* probe substrates for the major human hepatic sulfotransferase, SULT1A1, demonstrates improved selectivity with 2-aminophenol

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ABSTRACT

Sulfation, catalysed by members of the cytosolic sulfotransferase (SULT) enzyme family, is important in xenobiotic detoxification and in the biosynthesis and homeostasis of many hormones and neurotransmitters. The major human phenol sulfotransferase SULT1A1 plays a key role in chemical defence, is widely expressed in the body and is subject to a common polymorphism that results in reduced protein levels. Study of these enzymes *in vitro* requires robust probe substrates, and we have previously shown measurement of activity with the widely used SULT1A1 substrate, 4-nitrophenol, does not accurately reflect protein expression. Additionally, the high degree of substrate inhibition observed with this compound further reduces its value as a probe for SULT1A1. Here we show that 2-aminophenol is a more suitable probe substrate for quantifying SULT1A1 activity in human liver. This compound is sulfated at a high rate (V_{max} with purified recombinant SULT1A1 = 121 nmol/(min mg) and shows strong affinity for the enzyme (K_m with purified recombinant SULT1A1 = 9 μM) and, importantly, is a very poor substrate for the other major SULT1 enzyme expressed in liver, SULT1B1 (with V_{max} and K_m values of 17 nmol/(min mg) and 114 μM, respectively). Experiments with purified recombinant human SULTs and a panel of 28 human liver cytosols demonstrated that 2-aminophenol shows limited substrate inhibition with SULT1A1, and V_{max} values measured in liver cytosols correlated strongly with SULT1A1 enzyme protein levels measured by a quantitative immunoblot method. We therefore suggest that 2-aminophenol is a suitable substrate to use for quantifying SULT1A1 enzyme activity.

1. Introduction

Sulfation reactions are involved in the biosynthesis, metabolism and homeostasis of many endogenous chemicals (e.g. hormones and neurotransmitters), and in the detoxification and elimination of a plethora of xenobiotics such as drugs, dietary and environmental chemicals [1–3]. Normally the action of sulfation reduces biological activity and increases elimination; however occasionally sulfation results in a bioactivation step and may play an important role in chemical carcinogenesis [4].

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Abbreviations: SULT, sulfotransferase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate
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Sulfotransferases (SULTs) catalyse the transfer reaction between the near-universal sulfuryl donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) and the substrate, normally via a hydroxyl group on the substrate [1,5]. To date 13 expressed *SULT* genes have been identified in the human genome [6,7]. The human SULT1 family is involved in xenobiotic metabolism and detoxification and in the biosynthesis and homeostasis of steroids and neurotransmitters, and comprises four subfamilies (A, B, C and E) with at least eight isoforms (the *SULT1A3* and *SULT1A4* genes encode identical proteins) [2,6,7]. SULT1A1 is believed to be the most important SULT in xenobiotic metabolism because of its broad substrate preference, high affinity for many phenolic compounds, and wide tissue distribution throughout the body, and it is the major SULT expressed in the adult human liver (our unpublished work).

In order to study the function of individual isoforms of large enzyme families, probe substrates that faithfully reflect enzyme protein levels in tissue samples are necessary. Within the SULT1 family the high degree of substrate cross-talk between enzymes makes it extremely difficult to identify such isoform-specific probe substrates. For SULT1A3 dopamine at low micromolar concentrations is diagnostic, and 17β-estradiol at nanomolar concentrations is a good probe substrate for SULT1E1 [8]. However for SULT1A1 the widely used xenobiotic substrate 4-nitrophenol does not satisfy all the criteria for a good probe substrate, particularly due to the substantial substrate inhibition observed with this compound and large differences in kinetic profiles observed between individual human cytosol samples [9]. We also noted that 4-nitrophenol was extensively metabolised by SULT1B1, and therefore it was suggested that SULT1A1 activity could not be studied independently and accurately in tissue samples using 4-nitrophenol [9].

Here we have identified 2-aminophenol as a more appropriate probe substrate for SULT1A1, particularly in human liver cytosols. We have characterised the sulfation of 2-aminophenol by SULT1A1 and other relevant SULTs and compared it to 4-nitrophenol using both purified recombinant enzymes and a panel of human liver cytosols. The results indicate that 2-aminophenol is a valuable probe substrate for analysis of SULT1A1 activity in human liver *in vitro*.

2. Materials and methods

2.1. Materials

PAPS (>99% purity) was purchased from H. Glatt, German Institute for Human Nutrition, Potsdam, Germany, and PAP ^{35}S was from Perkin-Elmer, Beaconsfield, UK. All other chemicals were obtained form Sigma, VWR or Boehringer.

2.2. Cell-free extract preparation from bacteria expressing recombinant human SULTs

Recombinant human SULTs 1A1, 1B1, 2A1, 1E1, 1C2 and 1A3 were produced in *E. coli* as previously described [9–12]. Expression of recombinant protein in *E. coli* was induced with isopropyl-β-D-thiogalactopyranoside (100 μM) for 16 h. The protein was harvested by lysing the bacteria with 5 mL/g of BugBuster™ (Roche, Burgess Hill, UK) for 20 min followed by centrifugation at 21,000 × *g* for 35 min to remove debris. Lysates were aliquoted and stored at −80 °C until used (within 3 months).

2.3. Purification of SULTs from cell-free extracts

To obtain purified SULT enzymes recombinant *E. coli* cell-free extracts were subjected to sequential chromatography on anion exchange (Q-Sepharose) and affinity (3′,5′-ADP agarose) columns. The cell-free extract was mixed with ammonium sulfate (0.351 g/mL) for 1 h and then centrifuged at 16,000 × *g* for 15 min. The pellet was resuspended in 50 mM Tris/HCl buffer containing 1 mM 2-mercaptoethanol, pH 8. The solution was dialysed overnight to remove the salt and the protein sample loaded onto a Hi-Trap Q-sepharose column (Amersham Biosciences, Buckinghamshire, UK) attached to an ÄKTA purification system (GE Healthcare, Little Chalfont, UK). A gradient from 0 to 1 M NaCl over 70 mL was used to elute the protein in 2 mL fractions which were assayed for SULT activity. Fractions displaying activity were pooled and dialysed overnight then applied to a column of ADP agarose and bound protein was eluted from the column with 100 μM PAPS. Again fractions with activity were pooled, desalted on a PD-10 column (GE Healthcare) and their purity checked by SDS-PAGE before being aliquoted, flash frozen in liquid nitrogen and stored at −70 °C until use. All stages of protein purification were carried out at 4 °C.

2.4. Enzyme assays

The human liver samples used, and the preparation of cytosolic fractions from them by differential centrifugation, were as previously described [11]. Sulfation activity was determined using the PAP ^{35}S assay originally described by Foldes and Meek [13]. Reaction mixtures in a total volume of 160 μL were prepared containing: 0.1 M phosphate buffer (pH 7.4), purified SULT (1.5 μg SULT1A1, 2 μg SULT1B1 or 1 μg SULT1A3) or human cytosol (10 μg), substrate (0.01–600 μM depending on protein sample) and PAPS (20 μM) containing 0.09 μCi PAP ^{35}S. 2-Aminophenol (>98% purity) stock solutions were prepared fresh each day in 50% (v/v) aqueous ethanol and diluted in assay buffer prior to use. Reaction mixtures were incubated for 20 min in a circulating water bath at 37 °C, and were stopped by placing the samples on ice and adding 200 μL barium acetate (0.1 M). To remove unutilized PAPS 200 μL barium hydroxide (0.1 M) and 200 μL zinc sulfate (0.1 M) were added. The samples were mixed and centrifuged at 16,000 × *g* for 4 min. A sample (500 μL) of the resulting supernatant was removed and mixed with 4 mL scintillation fluid (Emulsifier safe). The mixture was then counted for 1 min per vial on a scintillation counter (Beckman Coulter, High Wycombe, UK). Data were analyzed using Excel (Microsoft) and Prism 4 (GraphPad Inc., San Diego, CA, USA) software.

2.5. Quantification of SULT1A1 by immunoblotting

Large SDS-PAGE gels (11% acrylamide monomer) were used to resolve protein samples which were transferred to PVDF membranes (Immobilon, Millipore, MA) for immunostaining

[14,15]. Membranes were blocked with 1% (w/v) BSA (Roche, UK) in 50 mM Tris, pH 7.9, 150 mM NaCl, Trition-X 100 (0.01%, v/v) (TBS-X) for 1 h. Sheep anti-SULT1A3 IgG [16] was used to analyse SULT1A1 as it cross-reacts with SULT1A1 and SULT1A3; however, SULT1A3 is not expressed in the adult human liver and the two enzymes can be easily resolved on SDS-PAGE [16]. Membranes were washed in TBS-X before being exposed to donkey anti-sheep IgG peroxidase conjugate in TBS-X containing 1% (w/v) BSA for 1 h. Finally the membrane was washed in TBS-X and developed with ECL reagents and Hyperfilm (GE Healthcare). Standard curves for SULT1A1 quantification were generated using between 1 and 20 ng of purified recombinant SULT1A1 and the quantity of human liver cytosolic protein was between 1.25 and 5 μg. Quantiscan 3.1 software (BioSoft, Cambridge, UK) was used to analyse the band densities of scans of the developed X-ray film made on a desktop scanner attached to a personal computer. Following pilot blots, the quantity of SULT1A1 in human liver cytosol samples was adjusted such that density of the bands were within the linear region of the standard curves on each blot.

2.6. Protein determination

The protein concentrations of the cell-free extracts, purified SULTs and human liver cytosols were determined using the Lowry method with BSA as the standard [17].

3. Results

3.1. Screen of substrates for activity with recombinant human SULTs

Initially we screened a set of 14 chemicals, mainly planar phenols known or predicted to be substrates for SULT1A1, for sulfation activity using recombinant SULT1A1 and SULT1B1 enzymes. Activity was first determined using *E. coli* cell-free extracts expressing recombinant SULT1A1 or SULT1B1 and substrate concentrations of 1, 10 and 100 μM (Fig. 1). In general SULT1A1 exhibited a higher activity towards each of the substrates than SULT1B1, consistent with previous findings (e.g. [12]), and the well known substrate inhibition phenomenon was also considerably more pronounced with SULT1A1. From these data, we could identify only a limited number of compounds that appeared to be potential substrates for sulfation by SULT1A1 but which were not also sulfated significantly by SULT1B1. From the group of compounds tested we chose 2-aminophenol to investigate further, since at concentrations of 1 and 10 μM, activity was at least 100-fold higher with SULT1A1 than SULT1B1. Another potentially suitable compound, 4-isopropylphenol, is known to be sulfated by SULT1A3 [18] and other SULTs at low concentrations, so was not considered a suitable candidate.

Next, to determine whether any of the other major SULT enzymes were able to sulfate 2-aminophenol, we tested the recombinant sulfotransferases SULT1E1, SULT1C2, SULT2A1 and SULT1A3 (as *E. coli* cell-free extracts) for sulfation activity with this compound. No significant 2-aminophenol sulfation activity was detected with SULT1E1, SULT1C2 or SULT2A1

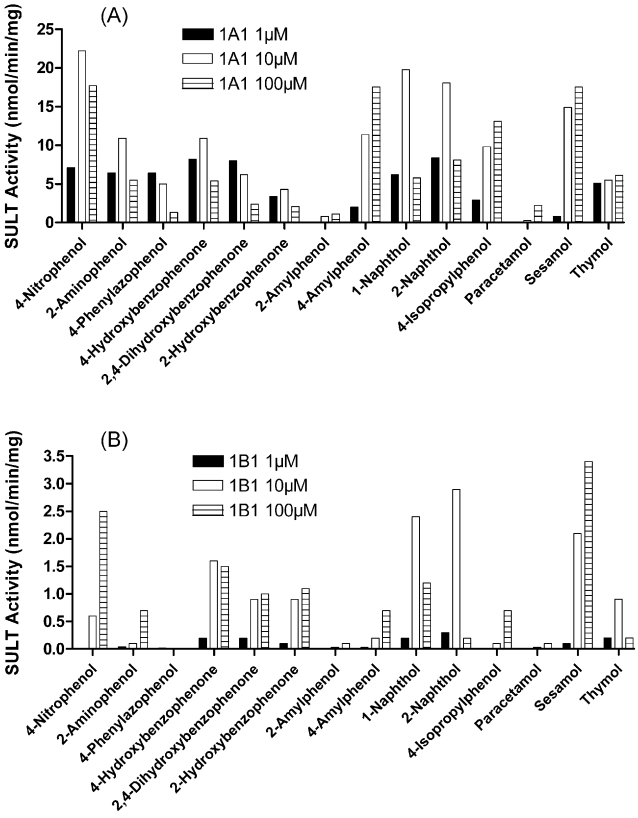

Fig. 1 – Substrate screen with recombinant human sulfotransferases. Sulfotransferase activity was measured with *E. coli* cell-free extracts expressing SULTs 1A1 (A) and 1B1 (B), with each substrate at three different substrate concentrations: 1 μM (filled bars), 10 μM (empty bars) and 100 μM (hatched bars). Data are mean of experiments carried out in duplicate.

(data not shown), although sulfotransferase activity was detected with known substrates for these enzymes (17β-estradiol, 4-nitrophenol and dehydroepiandrosterone, respectively), confirming that the enzyme preparations were functionally active. SULT1A3, however, did sulfate 2-aminophenol, although a much higher substrate concentration was required for maximal activity than for either SULT1A1 or SULT1B1. As SULT1A3 is not expressed in the adult liver [16], we felt that 2-aminophenol may still be a useful probe substrate for SULT1A1 in liver cytosol. This screen identified 2-aminophenol as a compound that was a very good substrate for SULT1A1 whilst being a poorer substrate for SULT1B1 and SULT1A3. Therefore we sought to investigate the sulfation of 2-aminophenol in more detail using purified recombinant human SULTs and human liver cytosols.

3.2. Sulfation of 2-aminophenol and 4-nitrophenol by purified human SULTs

As 2-aminophenol was identified as a potentially more selective substrate for SULT1A1 than 4-nitrophenol, a detailed examination of the sulfation of these two compounds was undertaken using purified recombinant enzymes. For human

Table 1 – Enzyme kinetic parameters for sulfation of 2-aminophenol and 4-nitrophenol by purified recombinant human SULTs

Purified SULT	2-Aminophenol				4-Nitrophenol			
	V_{max} (nmol/(min mg))	K_m (μM)	K_i (μM)	Intrinsic clearance (V_{max}/K_m)	V_{max} (nmol/(min mg))	K_m (μM)	K_i (μM)	Intrinsic clearance (V_{max}/K_m)
SULT1A1	121 ± 35	9 ± 5	108 ± 71	13.4	74 ± 27	4 ± 3	34 ± 24	18.5
SULT1B1	17 ± 1	114 ± 18	–	0.15	43 ± 11	65 ± 36	–	0.66
SULT1A3	121 ± 20	223 ± 86	–	0.54	69 ± 32	660 ± 487	–	0.10

Data are mean ± S.E.M. for measurements made in duplicate on three separate samples of purified protein.

SULT1A1, substrate inhibition is often seen with small phenolic molecules and multi-ring compounds such as 17β-estradiol [9,19,20]. Therefore in order to analyse the kinetic data obtained using SULT1A1 a modification of the Michaelis–Menten equation to reflect partial substrate inhibition was used ($Y = V_{max} X/(K_m + X(1 + X/K_i))$)—where K_i is the inhibition constant for the effect) whilst Michaelis–Menten kinetics ($Y = V_{max} X/(K_m + X)$) were assumed for SULT1B1 and SULT1A3. The V_{max} calculated for SULT1A1 with 2-aminophenol as substrate was 10-fold higher than for SULT1B1 (121 and 17 nmol/(min mg), respectively; Table 1), whilst the amount of substrate required to generate half the maximum rate (K_m) was 9 and 114 μM for SULT1A1 and SULT1B1, respectively, showing that significantly more substrate is required for SULT1B1 activity than SULT1A1. Moreover, the *in vitro* intrinsic clearance (V_{max}/K_m) for 2-aminophenol was some 90-fold higher for SULT1A1 than for SULT1B1, whilst 4-nitrophenol intrinsic clearance values only differed by a factor of 28. This again suggests that 2-aminophenol is a stronger and more selective substrate for SULT1A1 than 4-nitrophenol. The enzyme kinetics for purified SULT1A1, SULT1A3 and SULT1B1 show overlapping activity toward both substrates, particularly at concentrations above 10 μM (Fig. 2). However, with 4-nitrophenol there is more cross-talk between SULT1A1 and SULT1B1 (particularly at concentrations over 10 μM) than with 2-aminophenol as a substrate. With 2-aminophenol, SULT1A3 had higher activity than SULT1B1 at substrate concentrations greater than 10 μM. This suggested that in samples from tissues such as liver where a combination of SULTs is present, and in particular where SULT1B1 expression is prominent, 4-nitrophenol would be less likely to reflect solely the activity of SULT1A1 than 2-aminophenol.

3.3. *Sulfation of 2-aminophenol and 4-nitrophenol by human liver cytosol samples*

To determine whether 2-aminophenol could be used as a probe substrate for SULT1A1 in samples of human liver cytosol, we measured sulfation rates and calculated kinetic parameters for 2-aminophenol and 4-nitrophenol in 28 human liver cytosol samples (Fig. 3). The V_{max} and K_m values (mean ± S.E.M.) determined were 1.5 ± 0.1 and 1.8 ± 0.4 nmol/(min mg), and 2.0 ± 0.4 and 1.5 ± 0.5 μM for 2-aminophenol and 4-nitrophenol, respectively. Both substrates showed partial substrate inhibition kinetics, however this was far more dramatic with 4-nitrophenol ($K_i = 4.9 \pm 1.5$ μM) than with 2-aminophenol ($K_i = 79 \pm 18$ μM). As previously observed [9] there was variability in the extent of substrate inhibition between samples, with some samples showing total substrate inhibition at concentrations above 50 μM with 4-nitrophenol (not shown).

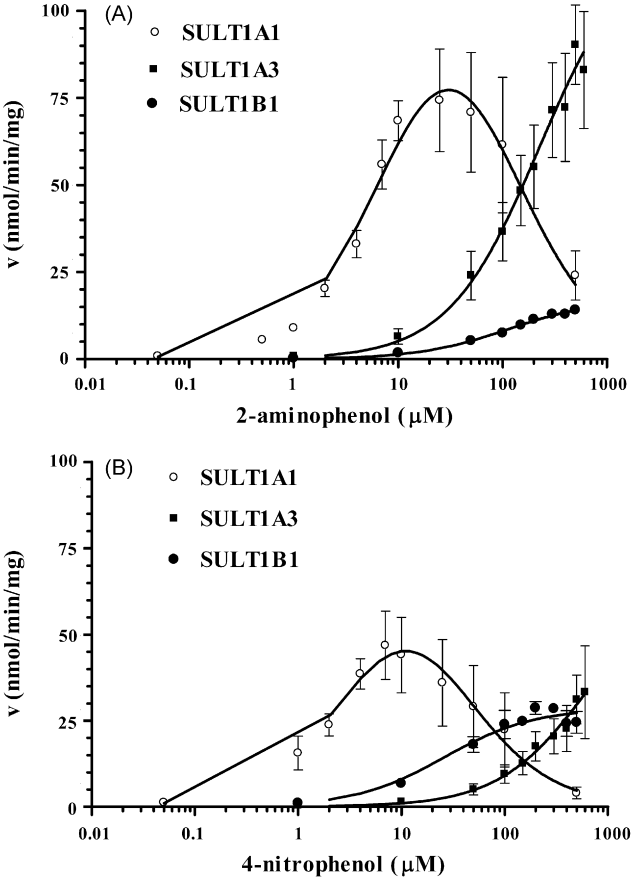

Fig. 2 – Sulfation of 2-aminophenol and 4-nitrophenol by purified recombinant human SULTs. Sulfation activity towards 2-aminophenol (A) and 4-nitrophenol (B) was measured over a range of substrate concentrations with purified recombinant SULTs 1A1 (○), 1A3 (■) and 1B1 (●). For SULT1A1, data were analysed using the equation for partial substrate inhibition $Y = V_{max} X/(K_m + X(1 + X/K_i))$, and for SULTs 1B1 and 1A3 with the Michaelis–Menten equation $Y = V_{max} X/(K_m + X)$. Data points represent the mean ± standard error for measurements performed in duplicate on three separate preparations of enzyme.

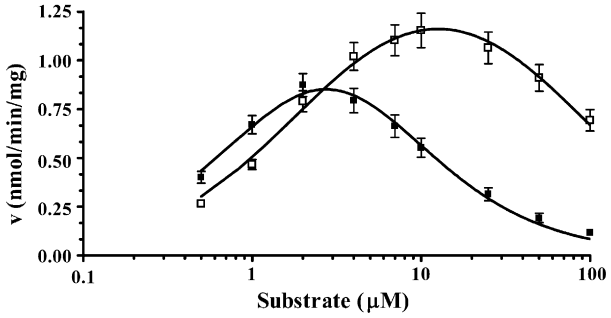

Fig. 3 – Sulfation of 2-aminophenol and 4-nitrophenol in human liver cytosol. Enzyme assays were carried out using cytosol fractions and a range of substrate concentrations using human cytosol samples and 2-aminophenol (□) or 4-nitrophenol (■) as substrate. Data were analysed using the equation for partial substrate inhibition (see legend to Fig. 2), and points represent the mean ± standard error of assays carried out in duplicate on a total of 28 liver cytosol samples.

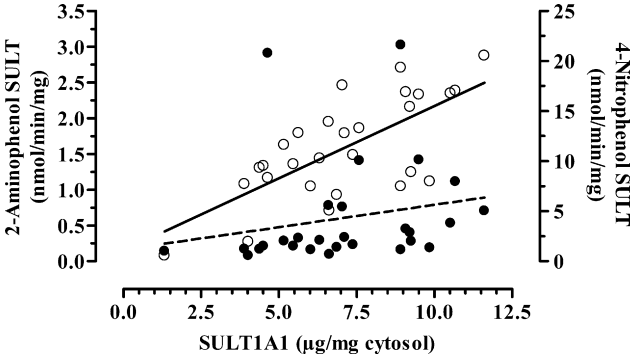

Fig. 5 – Correlation of SULT1A1 enzyme protein levels and enzyme activity in human liver cytosols. Sulfation activity (V_{max}) towards 2-aminophenol (○) and 4-nitrophenol (●) was compared to SULT1A1 protein levels using linear regression analysis. Pearson *r* values were 0.702 ($p < 0.0001$) and 0.203 ($p = 0.30$) for 2-aminophenol (indicated by solid line) and 4-nitrophenol (indicated by dotted line), respectively.

3.4. *Comparison of SULT1A1 quantity and probe substrate activity in human liver cytosol*

We next sought to determine whether the sulfotransferase activity measured with 2-aminophenol and/or 4-nitrophenol in human liver cytosol reflected the expression level of SULT1A1. The amount of SULT1A1 protein present in the liver samples was determined by a quantitative western immunoblot method developed in our laboratory [21] (Fig. 4). Purified SULT1A1 was used to generate standard curves on each separate blot, and the amount of SULT1A1 in individual human liver cytosol samples could be estimated by reference to these standard curves. Pilot experiments determined that the amounts of total cytosolic protein loaded onto the SDS-PAGE gels resulted in bands whose intensities fell within the range of the purified standard proteins (Fig. 4). The mean (±S.D.) SULT1A1 quantity for the 28 liver samples used was 7.1 ± 2.5 μg SULT1A1/mg cytosolic protein (range 1.32–11.61),

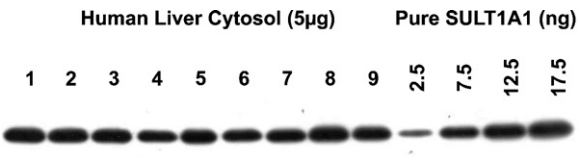

Fig. 4 – Quantification of SULT1A1 expression in human liver cytosols. Example of immunoblot used to quantify SULT1A1. Four samples of purified SULT1A1 ranging between 2.5 and 17.5 ng of protein were used to generate a standard curve specific to each blot, and 5 μg of human liver cytosol were loaded onto SDS-PAGE. Blots were probed with an antiserum raised in sheep against SULT1A3 that also detects SULT1A1, at a dilution of 1:30,000. The assays were optimized for protein load, exposure time and standard concentrations so that the signals were not saturated and band densities were within the linear range of the standard curve.

which translates to a mean of 168 μg SULT1A1/g of liver tissue. The quantity of SULT1A1 was then compared to sulfation rates of 2-aminophenol and 4-nitrophenol. When the V_{max} values of the two substrates were compared to SULT1A1 protein levels, a strong correlation was observed for 2-aminophenol (Pearson *r* value of 0.702, $p < 0.0001$), however there was no correlation with 4-nitrophenol sulfotransferase activity (Pearson *r* value of 0.203, $p = 0.30$) (Fig. 5). This most likely reflects the very wide differences in extent of substrate inhibition seen in human liver cytosol samples with 4-nitrophenol.

4. Discussion

In vitro probe substrates specific for individual members of multienzyme families such as the sulfotransferases are valuable experimental tools for studying their function. This has been employed to particularly good effect for the cytochrome P450 family (e.g. [22]) and to a lesser extent for the UGTs (e.g. [23]). For the SULT family this has proved particularly difficult due to the promiscuous nature of the substrate preferences for many of the enzymes, and the fact that in most tissues a number of different SULTs are expressed; therefore tissue cytosol preparations are usually a complex mixture of different isoenzymes some or all of which may have some activity toward the "probe" substrate.

The SULT1A1 enzyme is the most important xenobiotic-metabolizing member of the family in humans – it is the major SULT in the adult liver (our unpublished data), and has a very broad substrate profile, able to metabolise a host of phenolic compounds with low K_m values – usually <5 μM [12,18]. The compound 4-nitrophenol has been used for many years as a probe substrate for SULT1A1 [24], however we and others (e.g. [9]) have raised concerns regarding its suitability in this regard, particularly due to (a) a high level of cross-reactivity with the SULT1B1 enzyme and (b) the very strong substrate inhibition seen with SULT1A1-mediated sulfation of 4-nitrophenol,

which makes choice of substrate concentration particularly crucial. We therefore set out to identify a more suitable probe substrate for human SULT1A1.

The criteria for a suitable probe substrate were (a) having a high V_{max} for the SULT1A1 enzyme, both in purified form and in tissue cytosol preparations, (b) having a low K_m for SULT1A1, but not for other SULTs, (c) minimal turnover by other major SULTs and (d) minimal substrate inhibition kinetics. During the course of this study we identified 2-aminophenol as potentially a superior probe substrate than 4-nitrophenol for determining SULT1A1 activity in human liver cytosol samples. 2-Aminophenol shows a distinct preference for SULT1A1 over the other SULTs tested here. With purified recombinant SULT1A1 it demonstrated a low K_m and high V_{max}, and the resulting *in vitro* intrinsic clearance value was 90- and 24-fold higher than with SULT1B1 and SULT1A3, respectively, suggesting that in cytosol samples where a mixture of SULTs is present the majority of 2-aminophenol sulfation will be due to the action of SULT1A1, particularly at low substrate concentrations.

The analysis presented here also suggests that 2-aminophenol is a superior probe substrate for SULT1A1 compared with 4-nitrophenol. We have previously questioned the validity of 4-nitrophenol as a diagnostic substrate for SULT1A1 because of the large inter-individual variability in the extent of substrate inhibition seen in human liver cytosols and also its potential for metabolism by SULT1B1 [9]. Here we show that 4-nitrophenol was sulfated to a more significant extent by SULT1B1 than was 2-aminophenol. If, for example, a substrate concentration of 10 μM were used to compare activity (with purified recombinant enzymes, data from Fig. 2) the relative rates of 4-nitrophenol sulfation by the two enzymes would be 7:1 in favour of SULT1A1 over SULT1B1. In contrast, with 2-aminophenol SULT1A1 activity would exceed SULT1B1 activity by a factor of almost 40:1. This clearly suggests that measurement of 4-nitrophenol sulfation would be influenced to a greater extent by the presence of SULT1B1 in tissue cytosol samples than 2-aminophenol sulfation. When we compared activity towards the two substrates in liver cytosol samples a similar picture was seen. Significant substrate inhibition was observed when using 4-nitrophenol as the substrate (Fig. 3), with a K_i of 4.9 μM, meaning that at relatively low concentrations of 4-nitrophenol SULT activity is affected by this phenomenon. For 2-aminophenol there was only slight substrate inhibition observed (K_i = 79 μM). Therefore 2-aminophenol sulfation could be reliably measured over a much wider range of concentrations, implying that 2-aminophenol is a more robust substrate for quantifying SULT1A1 than 4-nitrophenol. This is especially important as there are known polymorphisms that affect SULT1A1 activity. A polymorphism in the *SULT1A1* gene, *SULT1A1*2* that codes for a single amino acid substitution $Arg_{213}His$ [25,26], results in reduced levels of SULT1A1 due to reduced protein stability [27]. We also showed that the wild type SULT1A1.1 protein exhibits a greater degree of substrate inhibition than the common variant allozyme SULT1A1.2 [9]. 2-Aminophenol may therefore be a more appropriate probe substrate for analysing the variations caused by this and other SULT1A1 polymorphisms.

To validate 2-aminophenol as a substrate that faithfully reflects SULT1A1 expression in tissue samples, the quantity of SULT1A1 in a panel of human liver cytosols was measured by immunoassay. As expected there was substantial variation in expression between the 28 individual cytosol samples. When we compared SULT1A1 expression level to enzyme activity measured with the two substrates, the calculated V_{max} values for 2-aminophenol correlated strongly with measured protein levels, whereas those for 4-nitrophenol did not. Thus the substrate inhibition characteristic of 4-nitrophenol with SULT1A1 again affected its reliability as a probe substrate, further supporting the applicability of 2-aminophenol in this regard.

Using recombinant proteins we showed that SULT1A3 was also able to sulfate 2-aminophenol, although the K_m was substantially higher for SULT1A3 (223 μM) than for either SULT1A1 (9 μM) or SULT1B1 (114 μM) suggesting that at low substrate concentrations interference from SULT1A3 would not be significant. Additionally, neither SULT1A3 enzyme protein nor enzyme activity (with probe substrate dopamine) can be detected in normal adult human liver cytosol, although it is expressed in the fetus [16]. Thus whilst 2-aminophenol is clearly a suitable probe substrate for SULT1A1 in liver cytosol, caution should be applied when using 2-aminophenol to investigate SULT1A1 activity in other human tissues, particularly where high levels of SULT1B1 and/or SULT1A3 are expressed such as the gastrointestinal tract. Indeed in a preliminary study with a small number of upper gastrointestinal tract cytosol samples biphasic kinetics were observed with 2-aminophenol (data not shown).

In conclusion we have shown that 2-aminophenol is a useful probe substrate for quantifying SULT1A1 enzyme activity in liver cytosols *in vitro*, and represents a considerable improvement over the presently accepted substrate 4-nitrophenol. In particular it exhibits substantially less substrate inhibition than 4-nitrophenol and is a less effective substrate for SULT1B1, the other major SULT1 family member expressed in liver. Thus 2-aminophenol may be useful in further understanding the role of SULT1A1 in humans.

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