Inhibitory effects of kynurenic acid, a tryptophan metabolite, and its derivatives on cytosolic sulfotransferases

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INTRODUCTION

Many xenobiotics and endogenous compounds such as neurotransmitters and steroid and thyroid hormones are metabolized through sulfate conjugation, which is catalysed by a family of SULTs (cytosolic sulfotransferases). The enzymes catalyse the transfer of a sulfonyl group from PAPS (3′-phosphoadenosine 5′-phosphosulfate) to an acceptor substrate compound containing either an hydroxy or amine group [1,2]. SULTs constitute a gene superfamily, which contains at least six different classes (i.e. SULT1–6 families in mammals) based on their identities of deduced amino acid sequences [2,3]. SULTs are often referred to as detoxification enzymes because in general sulfated molecules are highly soluble in water and easily excreted from the body. Since SULTs are important in the metabolism of endogenous as well as xenobiotic compounds, changes in the activity of these enzymes may have major implications for health.

Several xenobiotics, including environmental compounds, dietary components and drugs, exhibit strong inhibitory effects on SULTs. Sulfation of p-NP (p-nitrophenol) with hSULT1A1 (human SULT1A1; ST1A3) is significantly inhibited by flavonoids [4] and polychlorobiphenyls [5]. Polychlorobiphenyls have also been reported as potent inhibitors of thyroid hormone sulfation [6]. A number of non-steroidal anti-inflammatory drugs are shown to inhibit phenol SULTs. Among these drugs, mefenamic acid is the most potent inhibitor of hSULT1A1 in liver and duodenum cytosol [7]. Other drugs such as nimesulide, meclofenamate and piroxicam are also selective towards hSULT1A1 inhibition, whereas sulindac and ibuprofen exhibit more selective inhibitory effects on hSULT1E1 (ST1E4) sulfation [8]. In addition to xenobiotics, some endogenous compounds such as ATP [9], pyridoxal phosphate [10] and coenzyme A [11] could inhibit phenol SULTs. Because these endogenously produced inhibitors may modulate enzymatic activities in vivo, it is of great interest to identify such an endogenous inhibitor and to characterize them in terms of inhibitory potency and isoform specificity.

Recently, we have reported the involvement of SULT1B subfamily members in the sulfation of XA (xanthurenic acid) (Figure 1) to form natiuretic XA sulfate, and that XA is an excellent endogenous substrate for SULT1B members [12]. XA is a product generated in the kynurenine pathway which is the principle route of tryptophan catabolism [13]. In contrast with XA, an XA-related compound, KYNA (kynurenic acid) (Figure 1) exhibited the inhibitory effect on mSult1b1 (mouse Sult1b1; Stib3)-mediated sulfation of several compounds with low IC50 values [12]. KYNA is another metabolite produced during the kynurenine pathway and has a similar structure to XA, lacking an 8-hydroxy group [13].

KYNA has been reported as an endogenous glutamate antagonist in rodents [14] and primates [15]. Research into the...
glutamatergic antagonist effects of KYNA have led to the development of its derivatives as neuroprotectant drugs. Some KYNA analogues, including L695,902, L701,324, garvestinlel (GVI50526A), RPR104,632 and ZD9379, have progressed into clinical trials with the therapeutic indications of neuroprotection against brain damage resulting from ischaemia, hypoxia or traumatic brain injury [16].

KYNA is present not only in the central nervous system, but also in peripheral tissues. It has been reported that kynurenine aminotransferase I and II, which are responsible for the biosynthesis of KYNA from kynurenine, are widely distributed in most rat tissues [17]. This suggests a possible role of KYNA as a modulator of SULT activities in vivo because of its inhibitory effects on sulfation reactions. In the present study, we have investigated the inhibitory characteristics of KYNA and its derivatives on various recombinant human and mouse SULTs. The results demonstrate other distinct properties of KYNA as an inhibitor of hSULT1A1 and hSULT1B1 (ST1B2), as well as mSULT1B1. Similarly, some of the KYNA derivatives are also found to be SULT inhibitors.

**EXPERIMENTAL**

**Materials and animals**

Restriction endonucleases, TaKaRa Ex Taq and a DNA ligation kit were purchased from TaKaRaBio. The Thermo Sequenase cycle sequencing kit was obtained from PerkinElmer Life and Analytical Sciences. Ni-NTA (Ni\(^{2+}\)-nitrilotriacetic acid)-agarose was from Qiagen. An SDS/PAGE molecular mass standard was purchased from Bio-Rad. All other chemicals used were of the highest grade available. Oligonucleotides were synthesized at FASMAC Company. C57BL/6N mice (7 weeks old) were obtained from Charles River Japan. All animal experiments were carried out according to the guidelines of the Experimental Animal Welfare Committee of the Graduate School of Pharmaceutical Sciences, Tohoku University.

**Construction of expression plasmids for recombinant mouse sulfotransferases**

The coding region of mSult3a1 (St3a2; GenBank® accession no. NM_020565) was amplified by PCR from liver cDNA of female C57BL/6N mice, with oligonucleotides containing BamHI/HindIII sites (5'-GGCGATCCGATGACGATGACAAAATGG-3' and 5'-GCAAGCTTATCATCTCTTTTCGTC-3'). The PCR mixture (50 µl) contained 5 µl of the template cDNA, 10 pmol each of 5' and 3' primer, 0.2 mM each of dATP, dCTP, dTTP and dGTP, 0.25 units of TaKaRa Ex Taq and the Ex Taq buffer. After an initial denaturation at 94 °C for 3 min, the amplification was performed for 25 cycles of 1 min at 94 °C for denaturation, 30 s at 55 °C for annealing and 1 min at 72 °C for extension, and a final extension period of 4 min at 72 °C. The obtained cDNA fragment was ligated into a prokaryotic expression vector, pQE30 (Qiagen). The subcloned cDNA sequence was confirmed by direct sequencing with a DSQ2000-L DNA sequencer (Shimadzu Company). The constructed plasmid was introduced into Escherichia coli, M15(pREP4) strain (Qiagen). The amplified cDNA contained additional nucleotides in front of the initiation codon to include the amino acid sequence of an enterokinase recognition site (Asp-Asp-Asp-Asp-Lys).

**Bacterial expression and purification of recombinant sulfo transferases**

Recombinant His\(^{6}\)-SULT (hexahistidine-tagged SULT) was expressed and purified from the cytosol of E. coli with Ni-NTA-agarose as described previously [12]. In addition to mSult3a1, mSult2a1 (St2a4; [18]) was also expressed as a histidine-tagged protein and purified. Other SULTs including mSult1a1 (St1a4), mSult1b1, mSult1c2 (St1c9), mSult1d1 (St1d1), hSULT1A1, hSULT1A3 (ST1A5), hSULT1B1, hSULT1C2 (ST1C2), hSULT1E1 (ST1E4) and hSULT2A2 (ST2A3) were previously expressed and purified [12,19]. Protein concentrations of the recombinant enzymes were determined by spectrophotometry and calculated based on their molar absorption coefficient [20]. All of the purified proteins migrated as a single band corresponding to their predicted molecular mass and were at least 99% pure as evaluated by SDS/PAGE (Supplementary Figure S1 at http://www.BiochemJ.org/bj/422/bj4220455add.htm).

**Sulfation assay and kinetic analysis**

Sulfating activities were determined from the radioactivities of the metabolites obtained with \(^{35}\)SPAPS as a sulfate donor after TLC as described previously [12]. All enzyme assays were conducted under conditions where the linearity with respect to protein concentrations was obtained. A typical incubation mixture consisted of 50 mM Tris/HCl buffer [pH 7.4 or pH 10.0 (for the assay of desipramine)], 1 mM DTT, 5 mM MgCl\(_2\), 1 mg/ml BSA,
10 μM \(^{35}\text{S}\)PAPS, 2.5–50 ng of His-\(\alpha\)-SULT protein and various concentrations of substrates in a final volume of 10 μL of the reaction was initiated by the addition of \(^{35}\text{S}\)PAPS and terminated by the addition of 5 μL of ice-cold acetonitrile after incubation at 37 °C for 20 min. Aliquots (5–10 μL) of the reaction mixture were spotted on to a TLC plate (TLC aluminum plate silica gel 60 F254, 250 μm; Merck). The metabolites were developed with a solvent system of butan-1-ol/acetic acid/water (4:1:2, by vol.) or chloroform/methanol/water/28% ammonia aqueous solution (60:35:7.5:0.5, by vol.) (for the assay of DHEA, \(\beta\)-oestradiol and desipramine). The radioactive spots were analysed with an FLA-3000 image analyser (FujiFilm). The apparent kinetic parameters were obtained from the assays with several concentrations of XA (0.1–25 μM for mSult1b1; 0.5–75 μM for hSULT1B1 and 0.5–600 μM for others) [12], p-NP (0.01–1.5 μM for mSult1a1 and hSULT1A1; 2.5–500 μM for mSult1b1), T₃ (10–500 μM for mSult1b1 and 5–300 μM for hSULT1B1), DOPAC (2.5–250 μM), \(\beta\)-oestradiol (0.05–25 μM for hSULT1A1 and 1–1000 nM for hSULT1E1), DHEA (0.1–25 μM for mSult2a1 and 0.05–5 μM for hSULT2A1), desipramine (1–1000 μM), \(\alpha\)-naphthol (0.05–2.5 μM for hSULT1A1 and 0.1–5 μM for hSULT1B1) and dopamine (0.1–50 μM).

### Inhibition assay

To assess the inhibitory effect of KYNA, mefenamic acid, DCNP, DCKYNA, L689,560, L701,324 and gavestinel on SULT-mediated sulfations of various substrates, the reactions were performed as described above in the absence or presence of a wide-ranging concentration of each compound (KYNA, 0.25–250 μM; mefenamic acid, 0.1 nM–5 μM; DCNP, 0.001–500 μM; DCKYNA, 1–1000 μM; L689,560, 1–250 μM and gavestinel, 0.1–400 μM) or 10 μM L701,324. The substrate concentrations at their approximate apparent \(K_m\) values determined in kinetic studies were used for the assays.

### Data analysis

Apparent \(K_m\), \(V_{max}\) and IC₅₀ values were determined using the Prism 5 program (GraphPad Software).

### RESULTS

#### Enzymatic kinetics of SULT-mediated sulfations of various substrates

The kinetic characteristics of each mouse and human recombinant SULT were investigated with known respective substrates. The results are listed in Table 1. All of the sulfation reactions examined followed the typical Michaelis–Menten kinetics (results not shown). The apparent kinetic parameters were determined with a range of substrate concentrations where no substrate inhibition was observed. All mouse and human SULTs had no activity towards KYNA at a final concentration of 10 μM under the conditions used (results not shown).

#### Inhibitory effects of KYNA on SULTs

The influence of KYNA on SULT-mediated sulfations of various substrates was investigated. The results are summarized in Table 2. For mouse enzymes examined, KYNA exhibited preferential inhibitory effects on the sulfation of XA, p-NP and T₃, catalysed by mSult1b1 (Table 2 and Figure 2A) with IC₅₀ values in the low micromolar range (2.9–4.9 μM). For hSULTs, KYNA exerted the inhibitory activity towards both hSULT1A1 (Table 2 and Figure 2B) and hSULT1B1 (Table 2 and Figure 2C) to a similar extent, with IC₅₀ values of an order of magnitude greater than those for mSult1b1 sulfations. The values obtained for the inhibition of hSULT1A1-mediated sulfation of p-NP, \(\alpha\)-naphthol, \(\beta\)-oestradiol and XA were comparable with each other (18.8–31.8 μM), and so were the values for the inhibition of sulfoconjugation of p-NP, \(\alpha\)-naphthol, XA and T₃ catalysed by hSULT1B1 (19.6–52.9 μM).

#### Comparison of inhibition of SULTs by KYNA, mefenamic acid and DCNP

The inhibitory effects of KYNA was compared with those of DCNP, a non-specific SULT inhibitor, and mefenamic acid, the most potent known inhibitor of hSULT1A1 (Table 3). Substrate concentrations were set at roughly the \(K_m\) values determined in Table 1.

For mouse SULTs, DCNP showed the inhibitory effect on sulfation reactions catalysed by both mSult1a1 and mSult1b1, with greater potency for mSult1a1, in contrast with KYNA, which was identified as a selective inhibitor of mSult1b1. For human enzymes, DCNP inhibited the activity of both hSULT1A1 and hSULT1B1. As in the case of mouse enzymes, DCNP showed stronger effects on hSULT1A1 than hSULT1B1, whereas KYNA exhibited similar inhibitory potency for sulfation reactions mediated by hSULT1A1 and hSULT1B1. The IC₅₀ values for the inhibition of hSULT1A1-mediated p-NP and XA sulfation by DCNP were approx. two orders of magnitude lower than those for hSULT1B1-mediated T₃ and XA sulfation (Table 3).

Mefenamic acid exhibited very potent inhibitory activities towards both mouse and human SULT1A enzymes with IC₅₀ values...
values in the nanomolar range, whereas the effect on SULT1B enzymes was much weaker with IC<sub>50</sub> values of more than two orders of magnitude higher than those for SULT1A. The potency for inhibition of mSult1a1 was likely to be substrate-dependent. The IC<sub>50</sub> value for the inhibition of mSult1a1-catalysed p-NP sulfation was approx. 10-fold higher than that for XA sulfation. The inhibitory characteristics of mefenamic acid were completely different from those of KYNA, which exerted a selective inhibitory effect on mSult1b1 with very little effect on mSult1a1, and showed similar potency of inhibition for both hSULT1A1 and hSULT1B1.

### Inhibitory effects of KYNA analogues on SULTs

Because of antagonistic activities of KYNA against the glutamate receptor, it has been used as a lead compound for the development of neuroprotective drugs. We thus assessed the inhibitory effects of such drug candidates available, namely DCKYNA, L701,324, L689,560 and gavestinel (Figure 1), on SULT1A and SULT1B enzymes. As for KYNA, no sulfate metabolites of these compounds were detected with mouse and human SULT1A and SULT1B enzymes under the conditions used (results not shown). The inhibitory effects of DCKYNA, L689,560 and gavestinel were investigated and the results are presented in Table 3. The inhibitory potency of DCKYNA varied considerably depending on the combination of enzyme and substrate, with hSULT1A1 being the most susceptible enzyme. IC<sub>50</sub> values of DCKYNA for hSULT1A1 inhibition were comparable with those of KYNA inhibitions. On the other hand, L689,560 exhibited inhibitory activities towards hSULT1B1 with a potency similar to that of KYNA. The inhibitory characteristics of gavestinel were quite different from those of DCKYNA and L689,560. It exhibited potent inhibitory effects on hSULT1B1, as well as mSult1b1, with IC<sub>50</sub> values in the very low micromolar or submicromolar range. The potency of gavestinel for the inhibition of hSULT1B1 activity was approx. one order of magnitude greater than that of KYNA. Gavestinel also exerted strong inhibitory activities towards hSULT1A1 with a similar potency to KYNA and its IC<sub>50</sub> values were approx. one order of magnitude greater than those for hSULT1B1 sulfations. L701,324 showed no inhibitory activity at 10 μM towards mouse and human SULT1A enzymes with p-NP and XA as substrates and SULT1B enzymes with T<sub>3</sub> and XA as substrates (results not shown). We were unable to test a higher concentration of L701,324 because of its low solubility.

#### Mode of KYNA inhibition

The mode of inhibition by KYNA was examined for the sulfoconjugation of XA and p-NP catalysed by mSult1b1.
Table 3  Comparison of inhibitory effects of KYNA, DCNP, mefenamic acid and KYNA derivatives on sulfation reactions

Assays were performed as described in the legend to Table 1 in the absence or presence of 0.25–250 μM KYNA, 0.001–500 μM DCNP, 0.1 mM–5 μM mefenamic acid, 1–1000 μM DCKYNA, 1–250 μM L689,560 or 0.1–400 μM gavestinel, and 5 ng of recombinant enzymes. The values in parentheses are the substrate concentrations used (in μM). The data are expressed as the means ± S.D. from three independent determinations. *Values obtained from Table 2.

<table>
<thead>
<tr>
<th>SULT–substrate</th>
<th>IC_{50} (μM)*</th>
<th>DCNP (μM)</th>
<th>Mefenamic acid (nM)</th>
<th>DCKYNA (μM)</th>
<th>L689,560 (μM)</th>
<th>Gavestinel (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mSult1b1–XA (25 ng)</td>
<td>&gt;250</td>
<td>0.40 ± 0.02</td>
<td>899 ± 71.7</td>
<td>&gt;1000</td>
<td>&gt;250</td>
<td>135.5 ± 28.5</td>
</tr>
<tr>
<td>mSult1b1–p-NP (25 ng)</td>
<td>&gt;250</td>
<td>0.02 ± 0.00</td>
<td>75.6 ± 4.9</td>
<td>110.3 ± 11.7</td>
<td>194.3 ± 21.6</td>
<td>22.5 ± 2.0</td>
</tr>
<tr>
<td>hSULT1A1–XA (10 ng)</td>
<td>&gt;1000</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>196.8 ± 6.3</td>
<td>179.6 ± 14.1</td>
<td>0.72 ± 0.05</td>
</tr>
<tr>
<td>hSULT1B1–XA (10 ng)</td>
<td>18.0 ± 1.0</td>
<td>37.2 ± 1.0</td>
<td>&gt;500</td>
<td>20.8 ± 3.3</td>
<td>&gt;250</td>
<td>14.6 ± 2.8</td>
</tr>
<tr>
<td>hSULT1B1–p-NP (10 ng)</td>
<td>151.0 ± 34.0</td>
<td>14.9 ± 3.3</td>
<td>&gt;500</td>
<td>13.7 ± 2.1</td>
<td>117.0 ± 5.3</td>
<td>10.6 ± 0.3</td>
</tr>
<tr>
<td>hSULT1A1–p-NP (10 ng)</td>
<td>32.4 ± 3.4</td>
<td>32.4 ± 3.4</td>
<td>&gt;500</td>
<td>164.2 ± 10.8</td>
<td>53.6 ± 9.4</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>hSULT1B1–p-NP (10 ng)</td>
<td>34.2 ± 7.8</td>
<td>34.2 ± 7.8</td>
<td>&gt;500</td>
<td>253.6 ± 16.8</td>
<td>47.3 ± 3.4</td>
<td>1.7 ± 0.2</td>
</tr>
</tbody>
</table>

Table 4  Apparent kinetic parameters for sulfation reactions in the presence and absence of KYNA

Assays were performed as described in the legend to Table 1 in the absence or presence of KYNA at various concentrations with recombinant enzymes (2.5 ng for hSULT1A1-mediated p-NP sulfation and 5 ng for others). Values shown are means ± S.D. from three independent experiments (duplicate in each experiment).

<table>
<thead>
<tr>
<th>SULT–substrate</th>
<th>IC_{50} (μM)</th>
<th>K_{m} (μM)</th>
<th>V_{max} (nmol/mg of protein per min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mSult1b1–XA</td>
<td>0.45 ± 0.09</td>
<td>15.3 ± 0.3</td>
<td>24.6 ± 3.7</td>
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<tr>
<td>mSult1b1–p-NP</td>
<td>47.7 ± 3.7</td>
<td>229.8 ± 23.3</td>
<td>53.6 ± 2.4</td>
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<tr>
<td>hSULT1A1–XA</td>
<td>42.7 ± 2.5</td>
<td>44.6 ± 9.1</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>hSULT1B1–XA</td>
<td>55.9 ± 3.6</td>
<td>27.8 ± 3.2</td>
<td>26.3 ± 2.6</td>
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<tr>
<td>hSULT1A1–p-NP</td>
<td>0.35 ± 0.05</td>
<td>248.7 ± 30.4</td>
<td>26.3 ± 2.6</td>
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<tr>
<td>hSULT1B1–p-NP</td>
<td>0.17 ± 0.03</td>
<td>115.5 ± 10.3</td>
<td>26.3 ± 2.6</td>
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<tr>
<td>hSULT1B1–XA</td>
<td>0.11 ± 0.01</td>
<td>92.1 ± 5.3</td>
<td>26.3 ± 2.6</td>
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<tr>
<td>hSULT1B1–p-NP</td>
<td>0.063 ± 0.013</td>
<td>38.5 ± 1.2</td>
<td>26.3 ± 2.6</td>
</tr>
<tr>
<td>hSULT1A1–p-NP</td>
<td>8.6 ± 1.2</td>
<td>19.1 ± 1.5</td>
<td>26.3 ± 2.6</td>
</tr>
<tr>
<td>hSULT1B1–p-NP</td>
<td>18.2 ± 3.6</td>
<td>14.5 ± 0.6</td>
<td>26.3 ± 2.6</td>
</tr>
<tr>
<td>hSULT1A1–p-NP</td>
<td>26.1 ± 2.0</td>
<td>12.4 ± 1.3</td>
<td>26.3 ± 2.6</td>
</tr>
<tr>
<td>hSULT1B1–p-NP</td>
<td>39.8 ± 4.8</td>
<td>10.9 ± 0.3</td>
<td>26.3 ± 2.6</td>
</tr>
<tr>
<td>hSULT1B1–p-NP</td>
<td>30.7 ± 4.1</td>
<td>143.1 ± 5.8</td>
<td>26.3 ± 2.6</td>
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<tr>
<td>hSULT1B1–p-NP</td>
<td>43.9 ± 5.2</td>
<td>118.6 ± 4.9</td>
<td>26.3 ± 2.6</td>
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<tr>
<td>hSULT1B1–p-NP</td>
<td>52.8 ± 3.1</td>
<td>82.4 ± 1.8</td>
<td>26.3 ± 2.6</td>
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<tr>
<td>hSULT1B1–p-NP</td>
<td>61.0 ± 6.9</td>
<td>49.8 ± 2.9</td>
<td>26.3 ± 2.6</td>
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</tbody>
</table>

Novel inhibitors of cytosolic sulfotransferases

hSULT1A1 and hSULT1B1. The kinetic analyses were conducted in the absence or presence of various concentrations of KYNA with a fixed saturating concentration of PAPS (10 μM). The kinetics of all reactions were modelled using Michaelis–Menten equations. The kinetic parameters obtained from the analyses, together with the Lineweaver–Burk plots, were used to determine the mode of inhibition mediated by KYNA. Additionally, the data were also fitted to the enzyme inhibition models using the Prism 5 program to ascertain the most appropriate model of inhibition.

For the XA sulfation mediated by mSult1b1, hSULT1A1 and hSULT1B1, K_{m} values were increased and V_{max} values were decreased as the KYNA concentration was increased (Table 4 and Figures 3A, 3C and 3E). Lineweaver–Burk plots of the rate of XA sulfate formation against the XA concentration in the absence or presence of different concentrations of KYNA showed the pattern of mixed competitive/non-competitive inhibition (Figures 3A, 3C and 3E). The different mode of inhibition was observed for p-NP sulfation. In the case with mSult1b1 or hSULT1B1, Lineweaver–Burk plots of the rate of p-NP sulfation against the p-NP concentration converged at a single point on the x-axis (Figures 3B and 3F). Moreover, as the KYNA concentration was increased, there was a slight increase in K_{m} values and a great reduction in V_{max} values (Table 4), indicating non-competitive inhibition. Interestingly, unlike SULT1B enzymes, the mechanism of inhibition by KYNA of hSULT1A1-mediated p-NP sulfation was uncompetitive because both K_{m} and V_{max} values were obviously decreased along with an increase in the KYNA concentration (Table 4). Consistently, the plots between 1/V and 1/(p-NP concentration) in the absence or presence of different concentrations of KYNA generated straight lines in parallel (Figure 3D), which is the specific pattern of non-competitive inhibition.

**DISCUSSION**

KYNA is an endogenous product of tryptophan metabolism [13] and acts as an antagonist of ionotropic glutamate receptors [15,21]. In our previous study, the compound was identified as a potent inhibitor of SULTs [12]. Therefore, in the present study, we have characterized its inhibitory effects on several mouse and human recombinant SULTs. To this end, we have bacterially expressed and purified various SULTs, whose kinetic parameters have been determined to be consistent with previous reports [22–27]. Using these enzymes, we performed inhibition assays with KYNA. The results obtained demonstrate that KYNA is a *bona fide* inhibitor of SULTs and that it is selective for mSult1b1 among mouse enzymes tested, with an IC_{50} value within the low micromolar range. In the case for human enzymes, KYNA exhibited inhibitory activity towards hSULT1A1 as well as hSULT1B1, and the potency of inhibition for hSULT1A1 and hSULT1B1 was weaker than that for mSult1b1. Interestingly, KYNA derivatives, L689,560 and gavestinel, showed a higher potency for hSULT1A1 and hSULT1B1 inhibition than mSult1a1 and mSult1b1 (see below).

Various xenobiotics including environmental chemicals, therapeutic drugs and dietary compounds have been found to inhibit SULTs. A substance belonging to the group of hydroxylated polychlorinated biphenyls, 6′-OH-CB35, was shown to be a potent selective inhibitor of hSULT1B1 with an IC_{50} value of 4.7 μM [28], which is more potent than KYNA. The dietary chemicals cyanidin-3-rutinoside [29], catechin [29] and caffeic acid [30] are inhibitors of hSULT1A1 with a similar

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Figure 3 Mode of KYNA inhibition

Assays were performed at pH 7.4 with various concentrations of XA in the presence and absence of KYNA. Michaelis–Menten plots (left-hand side) and Lineweaver–Burk plots (right-hand side) with the y-axis as 1/(nmol/mg of protein per min) of various sulfation reactions are shown for mSult1b1-mediated XA sulfation (A), mSult1b1-mediated p-NP sulfation (B), hSULT1A1-mediated XA sulfation (C), hSULT1A1-mediated p-NP sulfation (D), hSULT1B1-mediated XA sulfation (E) and hSULT1B1-mediated p-NP sulfation (F). The kinetic parameters are summarized in Table 4. Each plot depicts the means of duplicate determinations in one typical experiment of three independent experiments.

KYNA has been shown to be present in several peripheral organs such as liver [17], kidney [17], heart [31] and retina [32], and is also detected in human serum with a concentration of 28.4 ± 2.16 nmol/l [33]. The human retina and vitreous body contain KYNA at 36.8 ± 7.6 and 33.1 ± 6.2 pmol/g of wet tissue weight respectively [32]. Based on these reports, the level of KYNA under normal conditions does not seem high enough to exert its inhibitory effect in vivo. However, increases in its levels have been reported in some diseases such as inflammatory neurological disease [34], schizophrenia [35] and chronic renal failure [36]. Thus it is of great interest to examine whether SULT enzyme activities in patients suffering from these diseases are different from those in healthy subjects.

The amounts of KYNA in the body may also be increased by the consumption of some drugs and foods. A recent study has demonstrated that memantine, a drug for Parkinson’s disease and other neurodegenerative disorders, can potently increase production of KYNA in brain cortical slices and mixed glial cultures via activation of a cAMP-related pathway [37]. A similar phenomenon could happen by the use of produgs of KYNA as glutamate antagonists and nicotinylalanine as a modulator to increase the level of KYNA in the body [38]. Furthermore, high concentrations of KYNA have been found in propolis, honey, bee pollen, fresh broccoli and potato [39]. KYNA is absorbed from the intestine and transported to the liver and kidney via the bloodstream [39]. Therefore consuming a large amount of these foods may lead to the decreased activity of SULT enzymes in several tissues.

KYNA has been used as a lead compound for the development of glutamate antagonists. In the present study, the inhibitory effects of KYNA derivatives, namely DCKYNA, L701,324, L689,560 and gavestinel, on SULT1A and SULT1B members was also investigated. Unlike KYNA, DCKYNA, which has chlorine-substitutions at the 5- and 7-positions of KYNA (Figure 1), exhibited preferential inhibitory effects on hSULT1A1 activity, but little inhibitory effect on hSULT1B1. L701,324 has a large bulky lipophilic substituent at the 3-position (Figure 1) and showed no inhibitory effect on SULT1A and SULT1B enzymes at 10 μM under the conditions used. Replacement of the 4-hydroxy group of KYNA with an amido-substituent leads to L689,560 (Figure 1). This compound exerted preferential inhibitory activity towards hSULT1B1 with a potency similar to that of KYNA. On the other hand, gavestinel, which has indole substituted instead of quinoline as a core structure (Figure 1), exhibited the very potent inhibitory effects on hSULT1B1 with IC₅₀ values in the low micromolar range. Collectively, the inhibitory spectrum of KYNA analogues depends on atom and/or side-chain substituents on the KYNA core structure, and/or the modification of the quinolone nucleus of KYNA to indole or other analogues.

In the present study, we have shown the potent inhibitory effects of gavestinel on hSULT1B1 and hSULT1A1. This compound is a
selective and potent antagonist at the glycine site of the NMDA (N-methyl-D-aspartate) receptor and has been investigated as a neuroprotectant in the rodent model of cerebral ischaemia [40]. The drug entered clinical trials in 1996 and the pharmacokinetics of gavestinel in patients with acute stroke was studied following an 800 mg loading dose and 100, 200 or 400 mg maintenance doses every 12 h for five doses [41]. The results showed that the peak plasma concentration was approx. 300 μM and the steady-state concentration was 50–230 μM [41]. These data suggest that gavestinel may exert its inhibitory effects on hSULT1B1, as well as hSULT1A1, in vivo. Moreover, it should be noted that, in addition to the above-mentioned KYNA derivatives including gavestinel, a series of compounds have also been developed from the KYNA structure and progressed into clinical trials [16]. Because hSULT1B1 and hSULT1A1 play important roles in the metabolism of thyroid hormones and β-oestradiol respectively, as well as drugs in humans, it remains to be examined in future studies whether such KYNA derivatives exhibit an inhibitory effect on SULTs in vivo.

The mode of KYNA inhibition for mSult1b1−, hSULT1A1- and hSULT1B1-catalysed p-NP and XA sulfations have been investigated in the present study. Kinetic analyses have revealed the mode of KYNA inhibition of XA sulfation mediated by these three enzymes to be mixed competitive/non-competitive. For this type of inhibition, Kᵢ (reflecting the binding of an inhibitor to a free enzyme) is less than Kᵢₑ (reflecting the binding of an inhibitor to an enzyme–substrate complex), which indicates that the binding of XA to SULT decreases the affinity of the enzyme for KYNA [42]. Other SULT inhibitors that have been demonstrated to exhibit mixed competitive/non-competitive inhibition include quercetin, which inhibits hSULT1A1-mediated p-NP sulfation [43], black tea and oolong tea, which inhibit hSULT1A3-mediated dopamine sulfation [44] and cigarette smoke toxicants, which inhibit hSULT1A1-mediated β-oestradiol sulfation [45]. The different modes of inhibition by KYNA were observed for p-NP sulfation. The kinetic studies showed that KYNA was an uncompetitive inhibitor of the p-NP sulfation mediated by mSult1b1 and hSULT1B1, whereas it demonstrated non-competitive inhibition with hSULT1A1. Similar results were observed for epigallocatechin gallate and epicatechin gallate, which were non-competitive inhibitors of hSULT1A1, whereas they exerted a mixed type of inhibition for hSULT1A2 and hSULT1A3 [46]. Collectively, the results obtained demonstrate that the mode of KYNA inhibition may vary depending on the individual substrate and enzyme involved.

In the present study, we observed a different susceptibility to KYNA inhibition between mSult1a1 and hSULT1A1, although they have similar kinetic characteristics for sulfations. The reason for this dissimilarity is at present unclear. Because the crystal structure is available only for hSULT1A1, but not mSult1a1, and there are several amino acid substitutions between hSULT1A1 and mSult1a1 (72% amino acid identity; [47]), further investigations such as X-ray crystallographic study of the complex of KYNA and enzymes and/or site-directed mutagenesis analyses will unveil the species-dependent inhibitory effect of KYNA.

In summary, in the present study we have demonstrated that KYNA, an endogenous metabolite of tryptophan, is a potent selective inhibitor of mSult1b1 and exhibits the inhibitory activity towards hSULT1A1 and hSULT1B1 with considerable potency. Moreover, a couple of derivatives of KYNA are also found to exert inhibitory activity towards SULTs. Among them, gavestinel is a very effective inhibitor for SULT1B1. Because SULT1B enzymes catalyse the sulfation of endogenous compounds such as thyroid hormones and XA as well as xenobiotics, the administration of gavestinel and other KYNA analogues may have consequences of reduced detoxification of xenobiotics and impaired homeostasis of endogenous compounds through the modulation of SULT activities.

AUTHOR CONTRIBUTION
Laddawan Senggunprai designed and performed the research, analysed the data and wrote the manuscript. Kouichi Yoshinari designed the research, analysed the data and wrote the manuscript. Yasushi Yamazoe assisted with analysing the data and preparing the manuscript.

ACKNOWLEDGEMENTS
We thank Dr Yoshimitsu Kakuta and Mr Takamasa Teramoto (Kyushu University, Fukuoka, Japan) for providing valuable information and discussion on the structure of SULTs.

FUNDING
This work was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Sciences and Technology of Japan; and the Comprehensive Research and Education Center for Planning of Drug Development and Clinical Education, Tohoku University 21st Century ‘Center of Excellence’ Programme.

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SUPPLEMENTARY ONLINE DATA

Inhibitory effects of kynurenic acid, a tryptophan metabolite, and its derivatives on cytosolic sulfotransferases

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Figure S1 SDS/PAGE of recombinant SULTs

Proteins (1 μg/lane) were resolved by SDS/PAGE (12 % gel) and stained with Coomassie Brilliant Blue G250. Lane 1, molecular mass standards; lane 2, mSult1a1; lane 3, mSult1b1; lane 4, mSult1c2; lane 5, mSult1d1; lane 6, mSult2a1; lane 7, mSult3a1; lane 8, hSULT1A1; lane 9, hSULT1A3; lane 10, hSULT1B1; lane 11, hSULT1C2; lane 12, hSULT1E1; lane 13, hSULT2A1.

Received 2 February 2009/April 2009; accepted 23 June 2009
Published as BJ Immediate Publication 23 June 2009, doi:10.1042/BJ20090168

The nomenclature of individual SULT (cytosolic sulfotransferase) forms is based on the Human Genome Nomenclature Committee (for human SULTs) and National Center for Biotechnology Information (for rodent SULTs). The names in the nomenclature system proposed previously by us [2,48] with an ‘ST’ prefix are also shown in the text. Their accession numbers are as follows: St1a4/mSult1a1, NM_133670; St1b3/mSult1b1, NM_019878; St1c9/mSult1c2, NM_026935; St1d1/mSult1d1, NM_016771; St2a4/mSult2a1, NM_001111296; St3a1/mSult3a1, NM_020565; ST1A3/hSULT1A1, NM_001055; ST1A5/hSULT1A3, NM_003166; ST1B2/hSULT1B1, NM_014465; ST1C2/hSULT1C2, NM_001056; ST1E4/hSULT1E1, NM_005420; ST2A3/hSULT2A1, NM_003167.

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