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Sequence Variants in Kynurenine Aminotransferase II (KAT II) Orthologs Determine Different Potencies of the Inhibitor *S*-ESBA

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The kynurenine pathway (KP, Figure 1) constitutes the major metabolic route of the essential amino acid L-tryptophan (L-Trp) in mammals. KP metabolism is initiated by oxidative opening of the indole ring of L-Trp and leads to the production of NAD^+. The catabolic intermediates of KP have recently attracted considerable attention for their role in central nervous system (CNS) physiology and in the etiology and progression of neurodegenerative and immunological disorders.[1–4] The major neuroactive metabolites originate from two competing branches of the pathway, where the central metabolite L-kynurenine (L-Kyn, **1**) is transformed into 3-hydroxykynurenine (3-OH-Kyn, **2**) and then further into quinolinic acid (QUIN, **3**), or into kynurenic acid (KYNA, **4**). KYNA is a competitive antagonist of the glycine site of the NMDA receptor complex, a non-competitive antagonist of the α7 nicotinic acetylcholine receptor,[5,6] and is endowed with neuroprotective properties. QUIN (**3**), in contrast, is a neurotoxic agonist of the NMDA receptor complex,[7,8] and 3-OH-Kyn (**2**) is a free radical generator that can contribute to neuronal damage in the CNS.[8]

In view of the neuroprotective or neurotoxic activities of KP metabolites, enzymes of the KP have long been considered interesting targets for rational therapeutic intervention.[3] One of the most promising targets is kynurenine aminotransferase (KAT), a pyridoxal-5′-phosphate (PLP)-dependent enzyme[9] that catalyzes the irreversible transamination of L-Kyn (**1**) into KYNA (**4**). Four isoforms of this enzyme have so far been identified, namely KAT I, KAT II, KAT III, and KAT IV (or mitAAT).[10,11] Although all four isoforms are present in the mammalian brain, the enzyme activity of KAT III has not been confirmed so far,[11] and only KAT I and KAT II have been thoroughly characterized with regard to their role in cerebral KYNA synthesis. These two isoforms differ in substrate specificity, with KAT I showing a lower specificity for L-Kyn (**1**) than KAT II.[10,12,13]

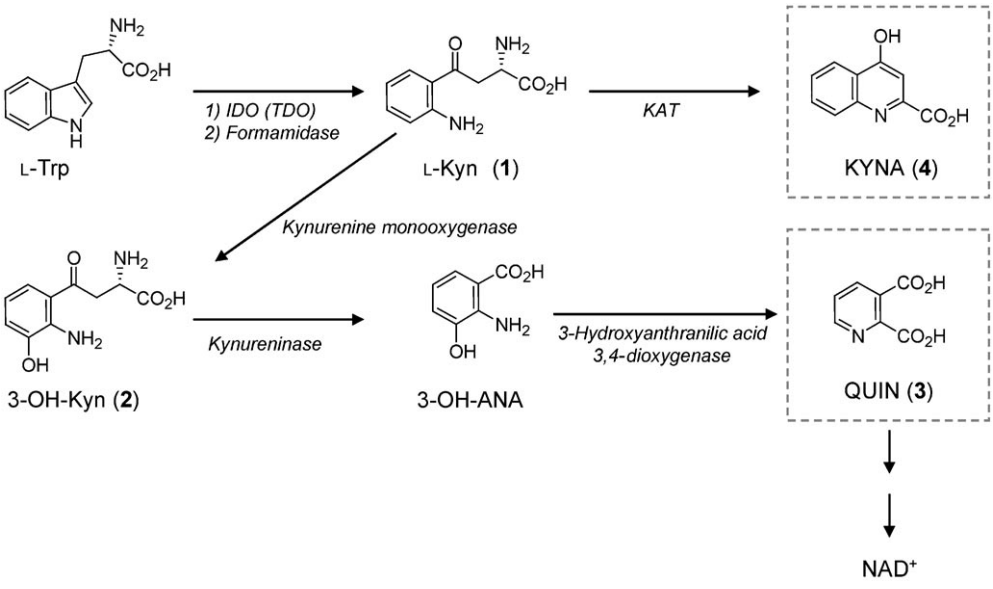

Figure 1. The kynurenine pathway (KP) of tryptophan metabolism.

The development of KAT inhibitors may prove useful in disorders associated with learning and memory deficits, where lowering the levels of brain KYNA may counterbalance glutamatergic and cholinergic hypofunction.[3,14] Recently, we reported the synthesis and biological characterization of (*S*)-4-ethylsulfonylbenzoylalanine (*S*-ESBA, **5**), the first potent and selective inhibitor of KAT II, which is the dominant KYNA-forming enzyme in the rat brain.[15] Biological assays conducted in vitro showed that *S*-ESBA (**5**) inhibits KAT II obtained from partially purified rat liver with an IC_{50} value of 6.1 μM. In vivo studies support these results, demonstrating that the administration of *S*-ESBA (**5**) lowers the extracellular levels of KYNA in the hippocampus of unanesthetized rats.[15]

Herein we report a novel and more efficient synthesis of *S*-ESBA (**5**) and an analysis of its inhibitory activity using purified recombinant human KAT II. The data are discussed in light of the crystal structure of human KAT II in complex with its natural substrate L-Kyn (**1**),[16,17] and on the basis of conserved and nonconserved residues that feature the sequences of species-specific orthologs of KAT II (human, rat, and mouse).

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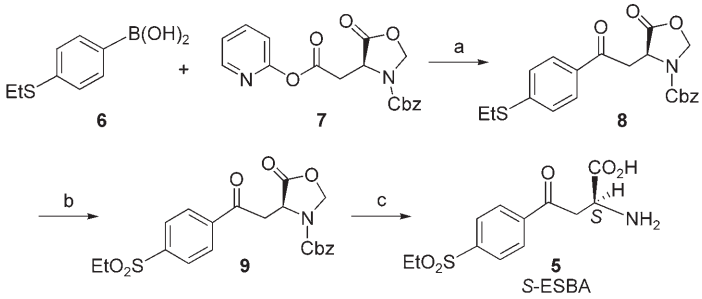
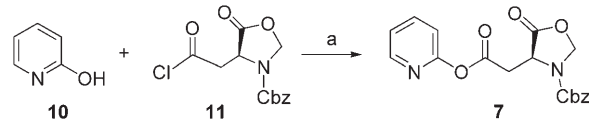
The first step of our previously reported synthesis of *S*-ESBA (**5**),[15] namely the conversion of 4-ethylthio-1-bromobenzene to the corresponding 4-arylstannane, suffered from a poor (28–35%) and inconsistent yield, and the use of highly toxic organotin compounds were another drawback in the synthetic route. We therefore developed a new and more efficient strategy for the preparation of **5**. Following a modified Suzuki–Miyaura reaction protocol,[18] we prepared the aryl ketone **8** by a palladium-catalyzed cross-coupling reaction of the 2-pyridyl ester **7** with commercially available 4-(ethylthio)phenylboronic acid (**6**) (Scheme 1). The preparation of (4*S*)-pyridin-2-yloxy-(3-carboxybenzyl-5-oxo-1,3-oxazolidin-4-yl)acetate (**7**) proved to be a critical step in light of the high instability of this derivative (Scheme 2). (4*S*)-(3-carboxybenzyl-5-oxo-1,3-oxazolidin-4-yl)acetic acid chloride (**11**)[19] was combined with 2-hydroxypyridine (**10**) in the presence of EDCI·HCl and DMAP, and, after fast filtration on silica gel, **7** was recovered in 65% yield and used immediately in the coupling reaction. Oxidation of the ethylthio substituent on the aromatic ring of **8** and final acidic hydrolysis completed the sequence, affording *S*-ESBA (**5**) in 77% overall yield.



Scheme 1. Reagents and conditions: a) $[Pd(OAc)_2]$, Ph_3P, dioxane, 50 °C, 12 h, 81% yield; b) *m*CPBA, $CHCl_3$, RT, 12 h, 95%; c) 1. 6 N HCl, 95 °C, 12 h, 2. Dowex 50W×2-200, 10% pyridine, 96%. *m*CPBA = *meta*-chloroperbenzoic acid.



Scheme 2. Reagents and conditions: a) EDCI·HCl, DMAP, CH_2Cl_2, 2 h at 0 °C then 12 h at RT, 65%. EDCI·HCl = 1-(3-dimethylaminopropyl)-3-ethyldicarbodiimide hydrochloride, DMPA = 4-dimethylaminopyridine.

To further characterize the biological properties of ESBA, the *S* enantiomer (compound **5**) was tested in vitro as an inhibitor of human and rat KAT II activity. KAT activity was measured as described by Guidetti et al.[13] Briefly, the reaction mixture contained a KAT II preparation (protein or tissue homogenate in 5 mM Tris-acetate buffer, pH 8.0, with 10 mM 2-thioethanol and 50 μM pyridoxal-5′-phosphate), [^{3}H]kynurenine (50 nCi; diluted with unlabeled kynurenine to a final concentration of 2 μM), 1 mM pyruvate, 70 μM pyridoxal-5′-phosphate, and 150 mM Tris-acetate buffer (pH 7.4) in a total volume of 200 μL. Solutions of aqueous *S*-ESBA (**5**) were added in aliquots of 20 μL immediately before initiating the assay; aliquots of the same volume of water were added in controls. Blanks were prepared by omitting the enzyme preparation. Samples were incubated for 2 h at 37 °C, and reactions were terminated by the addition of trichloroacetic acid (20 μL, 50% *w*/*w*) and HCl (1 mL, 0.1 M). The denatured proteins were removed by centrifugation, and newly synthesized [^{3}H]KYNA was eluted from Dowex 50W cation-exchange columns and quantified by liquid scintillation spectrometry.

Comparing human (recombinant and liver homogenate) and rat (partially purified and liver homogenate) preparations of KAT II, we found that **5**, as reported previously,[15] showed IC_{50} values of 6 and 72 μM toward partially purified and tissue homogenate, respectively, against rat KAT II. Unexpectedly, however, **5** was 10–20-fold less active as an inhibitor of the human enzyme (Table 1). Molecular docking experiments and sequence alignment analysis were undertaken to further examine the difference in inhibitory potency. To this end, the availability of the crystal structures of human KAT II both in complex with L-Kyn (PDB code: 2R2N)[16] and in the apo state (PDB code: 2QLR)[17] were exploited to gain insight into the key interactions between the inhibitor and the catalytic site of the enzyme. Because conformational rearrangements of PLP-dependent enzymes occur upon binding of substrates and inhibitors,[9] we deemed it advisable to use only the crystal structure of human KAT II in complex with L-Kyn (**1**)[16] for docking experiments of *S*-ESBA (**5**). Indeed, the structural similarity between L-Kyn (**1**) and *S*-ESBA (**5**) sustains the assumption that the inhibitor could adopt a conserved binding pose with the substrate in KAT II, and share a similar induced conformational fit of the catalytic site.

Table 1. Variance in potency of **5** as an inhibitor of rat (partially purified[12] and liver homogenate) and human (recombinant and liver homogenate) KAT II.[a]

S-ESBA (**5**) *c* [mM]	Inhibition [%]			
	rKAT II	hKAT II	rat LH	human LH
10	–	96±0	–	80±3
1	97±0	64±4	94±0	41±1
0.1	88±1	19±2	60±4	17±4
0.01	57±1	4±2	11±7	6±2

[a] Data are the mean ±SEM of three separate experiments and are expressed as percentage of control activity; LH = liver homogenate.

Briefly, a 3D model of *S*-ESBA was built using the Schrödinger package.[20] The atomic coordinates of the amino acidic and aromatic groups of *S*-ESBA (**5**) and L-Kyn (**1**) were mutually superimposed to produce the bioactive conformation observed in the co-crystallization experiments of the substrate and the enzyme. The obtained docking pose of **5** was energetically optimized using the Macromodel suite with the default minimization protocol and the OPLS 2005 force field.

In the resulting binding mode, while the carboxylic group of **5** forms a salt bridge with the guanidinium group of Arg 399,

the aromatic ring is engaged in a π–π stacking interaction with Tyr74 (Figure 2). Interestingly, the ethylsulfonyl moiety of *S*-ESBA occupies a narrow cavity defined by nine residues: Ile19, Arg20, Gly39, Leu40, Tyr74, Ser75, Pro76, Ser77, and Leu293.

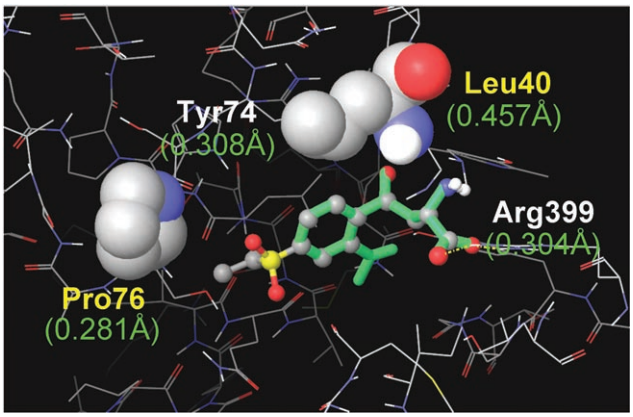


Figure 2. Binding mode of *S*-ESBA (**5**, ball and stick) built over the L-Kyn pose (tube green atoms) inside the binding site of KAT II complex (PDB code: 2R2N). Leu40 and Pro76 are shown in CPK style. Hydrogen bond interactions are highlighted in yellow. RMSD values (Å) of the residues between the substrate-bound state (PDB code: 2R2R) and the apo state (PDB code: 2QLR) of KAT II are given in brackets. The low values indicate that slight induced conformational rearrangements occur in the residues of the binding site.

A sequence alignment study (Figure 3) of the primary sequences of KAT II from the different species, namely mouse, rat, and human, was carried out using ClustalW server with the default options as implemented at the European Bioinformatics Institute (EBI).[21] The sequences were obtained from the National Center for Biotechnology Information (NCBI).[22] The alignment reveals that few nonconserved residues distinguish the polypeptide chains of the enzymes. Notably, in the rat ortholog of KAT II, both hydrophobic residues Leu40 and Pro76 of human KAT II are substituted by polar serine residues. By computationally mutating these residues and optimizing the interactions between *S*-ESBA (**5**) and the protein binding site, we obtained an interesting hydrogen bond formation between the sulfonyl oxygen atom and Ser76, and an overall different orientation of the ethyl moiety (Figure 4).

Although we cannot rule out that other nonconserved residues may indirectly affect different conformations of the catalytic site of diverse enzymatic KAT II orthologs, these two mutations may negatively influence the binding of *S*-ESBA (**5**) to human KAT II, providing steric clashes with the ethylsulfonyl moiety and divesting the sulfonyl group of pivotal hydrogen bond interactions.

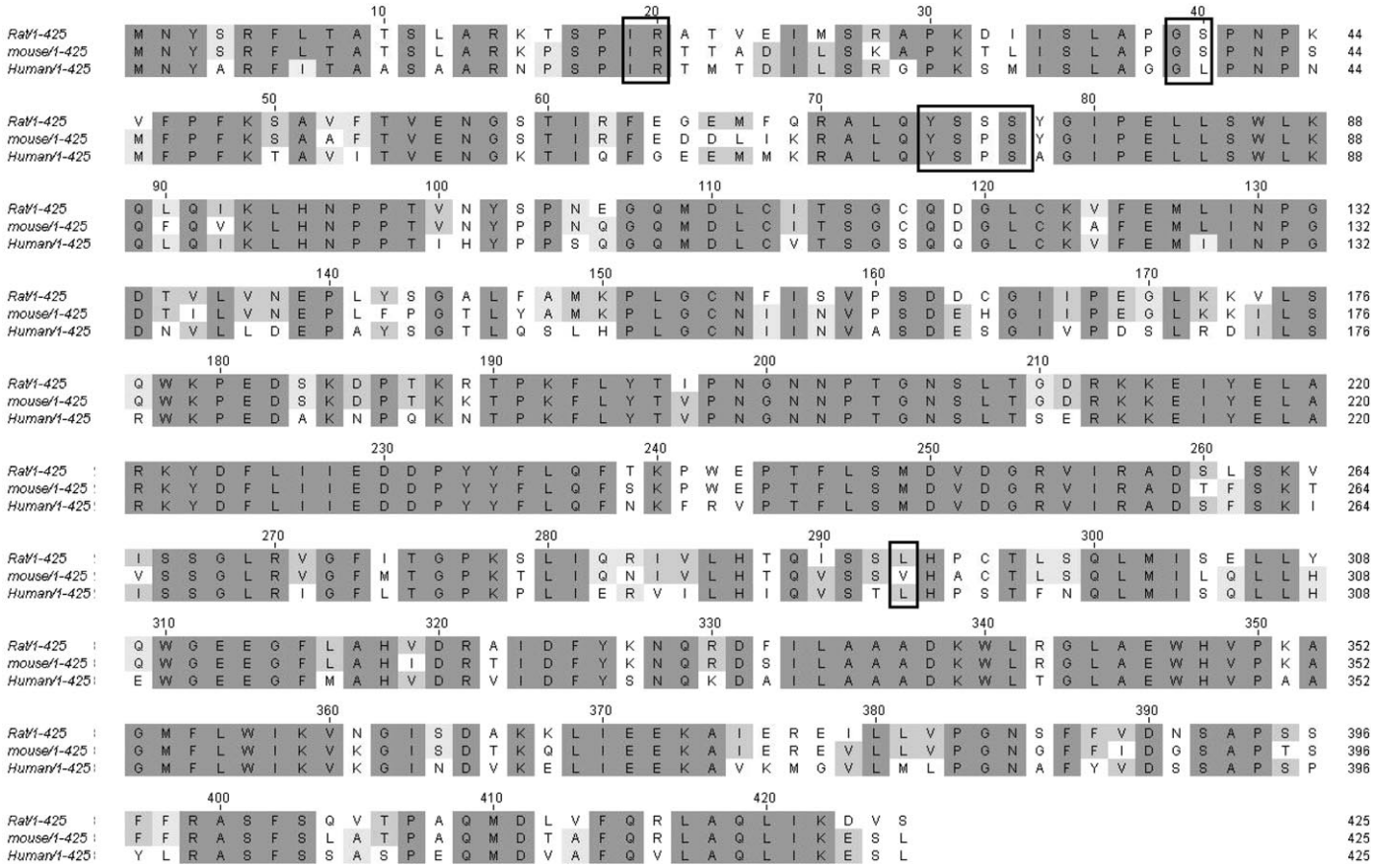


Figure 3. Alignment of KAT II sequences from three different species isoforms: rat, mouse, and human. The amino acids situated near the aromatic ring of L-Kyn in the crystal structure binding site (PDB code: 2R2N) are indicated with black boxes.

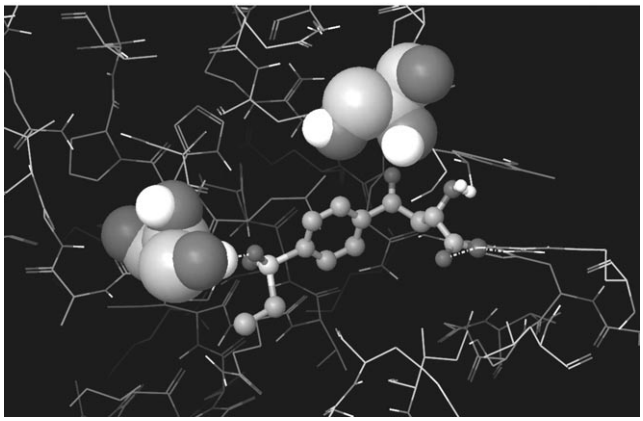


Figure 4. Binding mode of *S*-ESBA (**5**, ball and stick) inside the binding site of mutated KAT II crystal structure complex (PDB code: 2R2N). Both Leu 40 and Pro 76 are mutated to serines in the rat sequence, shown here in CPK style. Hydrogen bond interactions are highlighted.

In conclusion, our results demonstrate that sequence variants in human and rat KAT II diversely affect the inhibitory activity of *S*-ESBA (**5**), with **5** being less potent at inhibiting human KAT II than rat KAT II. We propose that this difference may arise from the presence of two mutations in the catalytic site, Leu 40 and Pro 76, the positions of which bear polar serine residues in the rat enzyme. Implications of our results for the development of clinically useful KAT II inhibitors are currently under investigation.

Acknowledgements

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Keywords: KAT II · kynurenic acid · kynurenine pathway · orthologs · sequence variants

[1] F. Moroni, *Eur. J. Pharmacol.* **1999**, *375*, 87–100.
[2] T. W. Stone, L. G. Darlington, *Nat. Rev. Drug Discovery* **2002**, *1*, 609–620.
[3] R. Schwarcz, R. Pellicciari, *J. Pharmacol. Exp. Ther.* **2002**, *303*, 1–10.
[4] M. L. Belladonna, P. Puccetti, C. Orabona, F. Fallarino, C. Vacca, C. Volpi, S. Gizzi, M. T. Pallotta, M. C. Fioretti, U. Grohmann, *Transplantation* **2007**, *84(Suppl. 1)*, S17–S20.
[5] M. Kessler, T. Terramani, G. Lynch, M. Baudry, *J. Neurochem.* **1989**, *52*, 1319–1328.
[6] C. Hilmas, E. F. Pereira, M. Alkondon, A. Rassoulpour, R. Schwarcz, E. X. Albuquerque, *J. Neurosci.* **2001**, *21*, 7463–7473.
[7] C. Rios, A. Santamaria, *Neurochem. Res.* **1991**, *16*, 1139–1143.
[8] P. Guidetti, R. Schwarcz, *Eur. J. Neurosci.* **1999**, *11*, 3857–3863.
[9] A. Amadasi, M. Bertoldi, R. Contestabile, S. Bettati, B. Cellini, M. L. Di Salvo, C. Borri-Voltattorni, F. Bossa, A. Mozzarelli, *Curr. Med. Chem.* **2007**, *14*, 1291–1324.
[10] P. Guidetti, L. Amori, M. T. Sapko, E. Okuno, R. Schwarcz, *J. Neurochem.* **2007**, *102*, 103–111.
[11] P. Yu, Z. Li, L. Zhang, D. A. Tagle, T. Cai, *Gene* **2006**, *365*, 111–118.
[12] R. Buchli, D. Alberati-Giani, P. Malherbe, C. Köhler, C. Broger, A. M. Cesura, *J. Biol. Chem.* **1995**, *270*, 29330–29335.
[13] P. Guidetti, E. Okuno, R. Schwarcz, *J. Neurosci. Res.* **1997**, *50*, 457–465.
[14] M. Varasi, A. Della Torre, F. Heidempergher, P. Pevarello, C. Speciale, P. Guidetti, D. R. Wells, R. Schwarcz, *Eur. J. Med. Chem.* **1996**, *31*, 11–17.
[15] R. Pellicciari, R. C. Rizzo, G. Costantino, M. Marinozzi, L. Amori, P. Guidetti, H. Q. Wu, R. Schwarcz, *ChemMedChem* **2006**, *1*, 528–531.
[16] Q. Han, Y. G. Gao, H. Robinson, J. Li, *Biochemistry* **2008**, *47*, 1622–1163.
[17] F. Rossi, S. Garavaglia, V. Montalbano, M. A. Walsh, M. Rizzi, *J. Biol. Chem.* **2007**, *283*, 3559–3566.
[18] H. Tatamidani, F. Kakiuchi, N. Chatani, *Org. Lett.* **2004**, *6*, 3597–3599.
[19] J. M. Scholtz, P. A. Bartlett, *Synthesis* **1989**, 542–549.
[20] Schrödinger, LCC, Maestro, **2007**, New York, NY, (USA).
[21] ClustalW: http://www.ebi.ac.uk/clustalw.
[22] NCBI: http://www.ncbi.nlm.nih.gov.

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