Inhibition of Kynurenine aminotransferase
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Chapter 1

Introduction:
The kynurenine pathway
1.1 Kynurenine pathway

Tryptophan is metabolized in several different ways (Figure 1). A physiologically important branch in the tryptophan metabolism is conversion into serotonin and melatonin. Up to 99% of tryptophan is metabolized in the kynurenine pathway (KP). However, until recently, this pathway was thought to be of merely catabolic importance. Nowadays, the regulatory role of this pathway is under investigation. Extensive research is being done to understand the effects of kynurenine pathway metabolites, shortly 'kynurenines', on biological systems.

The key intermediate of the kynurenine pathway is kynurenine (KYN). Two enzymes, indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), convert tryptophan into N-formylkynurenine. This intermediate is rapidly converted into KYN. At this point, the metabolic pathway splits up into two branches. One branch leads via 3-hydroxykynurenine (3-OH-KYN), anthranilic acid (AA) and 3-hydroxyanthranilic acid (3-OH-AA) to the endogenous NMDA receptor agonist quinolinic acid (QUIN). The second branch leads to the biosynthesis of kynurenic acid (KYNA), which has antagonistic properties on NMDA receptors and nicotinic acetylcholine receptors (nAChRs).

The ability to influence the exquisite balance between excitation and inhibition of neuronal systems through the kynurenine pathway can lead to new classes of pharmacological tools. These tools might be important in understanding processes in normal and diseased organisms on one hand, and in the clinical treatment in a number of diseases with major social impact on the other hand.

In general, QUIN has neurotoxic and KYNA has neuroprotective properties. Therefore, most research focuses on shifting the equilibrium within the kynurenine pathway away from QUIN and towards KYNA production. In a small number of cases, a shift away from KYNA production is desired. For example, elevated levels of this metabolite have been found in patients suffering from schizophrenia. Limiting brain levels of KYNA might also be beneficial for cognitive function. This thesis focuses on the characterization of the KYNA producing branch of the kynurenine pathway. This branch involves the enzyme kynurenine aminotransferase (KAT), which catalyzes the conversion of key intermediate KYN into KYNA. However, due to the highly balanced nature of the pathway, research will also relate to the first two enzymes of the QUIN branch of the pathway, kynureninase (KYNase) and kynurenine 3-hydroxylase (kynurenine mono-oxygenase, KMO).
Figure 1

Tryptophan metabolism. The kynurenine pathway is shaded grey.
1.1.1 Kynurenine production: Indole-2,3-dioxygenase, tryptophan-2,3-dioxygenase and kynurenine formamidase

Conversion of tryptophan to $N$-formylkynurenine is the rate limiting step in the kynurenine pathway. This reaction is catalyzed by indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase. 

Indoleamine 2,3-dioxygenase (IDO, EC 1.13.11.17) is found in extrahepatic tissues, including the brain. It has broad substrate specificity. IDO has protoheme IX as prosthetic group, and occurs in monomeric form. For metabolic activity, IDO needs superoxide or oxygen. The preferential use of superoxide gives the enzyme important antioxidative properties.\textsuperscript{1,6-13}

The immune system plays an important role in the regulation of IDO activity. IDO is induced by lipopolysaccharides, IFN-$\gamma$, TNF-$\alpha$, and IFN-$\alpha$ and other immuno-related compounds. Anti-inflammatory cytokines, such as IL-4 and TGF-$\beta$, are able to suppress IDO activity.\textsuperscript{1} Immune activation in mice caused induction of IDO activity and elevation of QUIN brain and plasma levels. These effects were attenuated by immune suppressors, like dexamethasone.\textsuperscript{14} However, high doses of glucocorticoids (hydrocortisone, dexamethasone) were found to enhance IFN-$\gamma$ mediated IDO activity in human cell lines.\textsuperscript{15} Differences between the studies could be explained by action of the glucocorticoids on different immunomodulatory systems.

IDO activity also closely relates to the nitric oxide pathways. NO inhibits IDO. Inhibition of nitric oxide synthetase results in elevated tryptophan to KYN turnover by IDO.\textsuperscript{1}

Competitive substrates, e.g. 6-chloro-$D,L$-tryptophan and 4-chloro-3-hydroxyanthranilic acid\textsuperscript{14}, 1-methyl-$D,L$-tryptophan, and the benzofuranyl- and benzo(b)thienyl analogs of tryptophan\textsuperscript{16} are known synthetic IDO inhibitors. Exiguamine A is a potent inhibitor ($K_i = 210$ nM) isolated from the sponge Neopetrosia exigua.\textsuperscript{17}

Induced IDO activity and resulting elevated levels of QUIN are important targets for the treatment of immune related diseases, like HIV, cardiovascular diseases and allergies.\textsuperscript{18-22}

Tryptophan 2,3-dioxygenase (TDO, EC 1.13.11.11) is the primary tryptophan metabolizing enzyme in the liver. TDO selectively metabolizes $L$-tryptophan, and uses oxygen in its catalytic cycle. TDO naturally occurs as a tetramer, and has one heme group in each monomer.\textsuperscript{12} TDO is inducible by dexamethasone in mice. QUIN levels are not elevated by TDO induction, probably due to extensive KYN metabolism into other metabolites in the liver.\textsuperscript{14} TDO activity is inhibited
Introduction: The kynurenine pathway

Kynurenine formamidase (arylformamidase, AFMID, EC 3.5.1.9) catalyzes the conversion of \( N \)-formylkynurenine to \( L \)-KYN.\(^{27, 28}\) The enzyme is also capable of processing some other amides and esters.\(^{27}\) AFMID is a member of the serine hydrolases. In mice, AFMID is present in most tissues, but the activity is highest in liver and kidney.\(^{29}\) A brain specific formamidase was found in some species.\(^{30}\) AFMID knock out mice showed kidney failure and disturbances of the immune system. Brain function was not affected.\(^{29}\) AFMID activity is modified by organophosphates present in insecticides. These compounds inhibit the enzyme by coupling to a serine residue in the active site. The effect of organophosphate diazinon on KYN pathway metabolites was studied best.\(^{31, 32}\) Diazinon was able to increase \( N \)-formylkynurenine and decrease KYN levels in mouse liver in vivo. Unexpectedly, plasma KYN levels were increased. Moreover, elevated levels of plasma QUIN, as well as increased renal KYNA and xanthurenic acid (XA) excretion were found. The unexpected increases of these kynurenine metabolites were explained by a secondary mechanism for KYN level regulation by release from unidentified storage.\(^{31}\)

1.1.2 Kynurenine

\( L \)-Kynurenine (KYN) is transported over the blood brain barrier by the large neutral amino acid transporter.\(^{33}\) Elevated KYN levels result in higher conversion rates into 3-OH-KYN, QUIN and KYNA, depending on available enzymatic systems. The physiological function of KYN is thought to be rather limited. One study showed an induction of nerve growth factor production in cultured mouse astrocytes by \( L \)-KYN.\(^{34}\) KYN is metabolized into two different directions. The compound is converted to KYNA by kynurenine aminotransferases (KATs) and into QUIN by conversion into the intermediates 3-OH-KYN and AA by kynurenine 3-hydroxylase (KMO) and kynureninase (KYNase), respectively. The conversion into either route is highly balanced. The balance can be shifted by inhibition of the KYN converting enzymes. For the design of enzyme inhibitors, the chemical structure of KYN is used extensively as a basis.
1.1.3 Kynurenine 3-hydroxylase

*Kynurenine 3-hydroxylase* (kynurenine monooxygenase, KMO, EC 1.14.13.9) is responsible for conversion of key intermediate KYN into 3-OH-KYN. The enzyme is a NADPH dependent flavin monooxygenase (FMO). Recombinant human KMO has 486 amino acid residues and has a weight of 56kDa. The enzyme has an optimal activity at pH 7.5. The mechanism of monooxygenation involves one molecule of NADPH and one non-covalently bound molecule of FAD. The active site properties indicate close resemblance to *p*-hydroxybenzoate hydroxylase.

Human KMO shows high selectivity for *L*-KYN. AA, KYNA, *L*-Trp, *D*-KYN, and *D*-hydroxybenzoate are no KMO substrates. KMO is competitively inhibited by pyridoxal 5′-phosphate and Cl− ions.

KMO is primarily localized on the outer membrane fraction of mitochondria. Rat KMO activity is highest in liver and kidney. In the brain, the activity was found to be 0.1% of liver activity. 3-OH-KYN has neurotoxic properties starting at a concentration of 1 µM. The neurotoxicity is caused by oxidation of the compound and concomitant production of reactive oxygen species (ROS), including superoxide radicals and hydrogen peroxide. The ROS formed are able to cause apoptosis. Antioxidants can inhibit 3-OH-KYN toxicity. Prior to being oxidized, 3-OH-KYN is transported into neurons by Na+ dependent neutral amino acid transporters. The different
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vulnerability of brain regions (cortex > striatum > hippocampus > cerebellum) can be explained by availability of these transporters. \(^{40}\)

**KMO inhibitors**

Inhibition of KMO has been studied extensively. Structures of most known KMO inhibitors are closely related to the enzymatic substrate KYN. A selection of published KMO inhibitors is listed in Table 1.

**Table 1 Selection of published KMO inhibitors**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC(_{50}) or % inhibition</th>
<th>Enzyme source(^{5})</th>
<th>Effect on other enzymes</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>nicotinylalanine</td>
<td>900±180 µM</td>
<td>liver, brain</td>
<td>KYNase: IC(_{50}) 800+/−120 µM</td>
<td>41</td>
</tr>
<tr>
<td>m-nitrobenzoylalanine (mNBA)</td>
<td>0.9±0.1 µM</td>
<td>brain, liver</td>
<td>KYNase: IC(_{50}) 100±12 µM</td>
<td>41, 42</td>
</tr>
<tr>
<td></td>
<td>3.0±0.2 µM</td>
<td>brain</td>
<td>&lt;50% inhibition at 100 µM for KAT and KYNase</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>0.77±0.11 µM</td>
<td>kidney</td>
<td>(S): IC(_{50}) 100 µM for QUIN formation in human monocyte-derived macrophages</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.8 µM</td>
<td>liver</td>
<td>KYNase: IC50 100 µM</td>
<td>46</td>
</tr>
<tr>
<td>3,4-dichlorobenzoylalanine (FCE28833, PNU156561)</td>
<td>0.2 µM</td>
<td>brain</td>
<td>KAT IC50&gt;1000 µM, KYNase IC50&gt;100 µM</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>0.2±0.02 µM</td>
<td>forebrain</td>
<td>&lt;50% inhibition at 100 µM for KAT and KYNase</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>0.23±0.032 µM</td>
<td>kidney</td>
<td>(S): IC(_{50}) 0.7 µM for QUIN formation in cultured human monocyte derived macrophages</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.33±0.03 µM</td>
<td>liver</td>
<td>KAT IC50&gt;1000 µM, KYNase IC50&gt;100 µM</td>
<td>48, 49</td>
</tr>
<tr>
<td></td>
<td>0.20±0.02 µM</td>
<td>brain</td>
<td>IC50 0.7 µM for QUIN formation in cultured human monocyte derived macrophages</td>
<td></td>
</tr>
<tr>
<td>4-(3-chlorophenyl)-2-hydroxy-4-oxo-but-2-enoic acid methyl ester</td>
<td>1.9 µM</td>
<td>liver</td>
<td>KAT in rat brain: 5 ± 6%@0.1 mM 4 ± 0%@0.01 mM 4 ± 1%@0.001 mM</td>
<td>50, 51</td>
</tr>
<tr>
<td></td>
<td>0.006 µM</td>
<td>human monocyte-derived macrophages</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>UPF 648</td>
<td>0.02 µM</td>
<td></td>
<td></td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>0.040 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100±9%@0.1 mM, 92±7%@0.01 mM</td>
<td>brain</td>
<td>KAT in rat brain: 5 ± 6%@0.1 mM 4 ± 0%@0.01 mM 4 ± 1%@0.001 mM</td>
<td>53</td>
</tr>
</tbody>
</table>
Table 1 (continued)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Km (µM)</th>
<th>Tissue</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-chloro-3-methyl-1H-pyrrolo[3,2-c]quinoline-4-carboxylic acid</td>
<td>24.0±3.1 µM</td>
<td>liver</td>
<td>KAT: 24% at 100 µM; KYNase: 25% at 100 µM</td>
</tr>
<tr>
<td>Ro 61-8048</td>
<td>8.1±1.2 µM</td>
<td>brain</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.037±0.003 µM</td>
<td>kidney</td>
<td></td>
</tr>
</tbody>
</table>

*All values were obtained from rat tissue homogenates, unless stated otherwise.

Two of the first compounds tested as KMO inhibitors were nicotinylalanine and m-nitrobenzoylalanine (mNBA).\(^{41, 42, 54}\) 3,4-Dichlorobenzoylalanine (FCE 28833, PNU 156561) was included into several studies focusing on inhibitory properties of KYN analogs. In 1996, it was published as part of a series of 2-, 3-, and 4-substituted benzoylalanines (Figure 2).

Figure 2

2,3,4-substituted benzoylalanines

4-phenyl-4-oxo-butanoic and -butenoic acids

pyrrolo[2,3-c]quinolines

bisphenol A

*Classes of KMO inhibitors*

The compound was shown to be selective for KMO over KYNase and KAT *in vitro*.\(^{47}\) The study was extended with several other KYN analogs, having 4-phenyl-4-oxo-butanoic and 4-phenyl-4-oxo-butenoic backbones (Figure 2). The α-hydroxy- and α-benzyl analogs of FCE 28833 were almost equally potent.\(^{49}\) The 4-phenyl-4-oxo-butenoic acid series was further extended by the group of
Rainhard. The most potent compound of this series, 4-(3-chlorophenyl)-2-hydroxy-4-oxo-2-enoic acid methyl ester (Table 1), had an IC\textsubscript{50} as low as 6 nM at QUIN formation in human macrophages, but had a much weaker effect on KMO in tissue homogenate.\textsuperscript{37,45}

A structurally slightly more restricted inhibitor is UPF 648. The compound is KMO over KAT selective at nanomolar concentrations.\textsuperscript{50,51,53}

An example of an inhibitor that is structurally not closely related to KYN is 7-chloro-3-methyl-1\textsubscript{H}-pyrrolo[3,2-c]quinoline-4-carboxylic acid (Table 1). This compound was studied in a series of pyrrolo[3,2-c]quinoline derivatives (Figure 2). Although the compound is less potent than KYN analogs, it shows good KMO over KYNase and KAT selectivity.\textsuperscript{48}

A more extensively used structurally diverse KMO inhibitor is Ro 61-8048 (Table 1). This compound shows high KMO inhibitory potency \textit{in vitro} and \textit{in vivo}.\textsuperscript{44}

Finally, the metabolic disruption of bisphenol A (Figure 2), a commonly used monomer of polycarbonate plastics, was attributed to inhibition of KMO.\textsuperscript{55}

\textit{In vivo} effects of KMO inhibitors on KYNA brain production have been studied extensively. In mice, i.p. administration of UPF 648 caused an increase of KYN and KYNA in striatal brain tissue.\textsuperscript{52} In rat, coinjection in striatum of UPF 648 with \textsuperscript{3}H-KYN caused a decrease of \textsuperscript{3}H-3-OH-KYN recovery from striatal brain tissue compared to injection of \textsuperscript{3}H-KYN alone. Tritiated QUIN and KYNA recovery were not changed in these experiments by UPF 648.\textsuperscript{53} mNBA (i.p.) caused increased KYN\textsuperscript{41,42} and KYNA\textsuperscript{41,42,46} concentrations in rat hippocampus, as measured by microdialysis. Whole brain content of KYN and KYNA were also increased.\textsuperscript{41,46} In mice, administration of KYN (i.p.), followed by oral administration of mNBA caused a dose-dependent increase of KYNA and a concomitant dose-dependent decrease of QUIN in whole brain tissue.\textsuperscript{45} Oral\textsuperscript{43,44} or intraperitoneal\textsuperscript{43} administration of FCE 28833, as well as administration of Ro 61-8048 (p.o.)\textsuperscript{44} caused increased extracellular KYNA levels in rat hippocampus.

Endotoxin stimulated excessive QUIN production in rat cortical tissue could be antagonized by KMO inhibitor nicotinylalanine.\textsuperscript{54} In these studies, effects of the inhibitors on 3-OH-KYN and AA were not taken into account.

Local infusion in rats of KYNA (100 nM) resulted in decreased neuronal activity, marked by reduced extracellular levels of glutamate (Glu) in the cortex and the caudate, but not in the hippocampus. Interestingly, whereas Ro 61-8048 was able to elevate extracellular KYNA levels in rat cortex, caudate and hippocampus, expected concomitant decrease in extracellular glutamate levels was only seen in the caudate.\textsuperscript{56} KMO inhibitors were shown to have sedative and anticonvulsive properties in rats and mice.\textsuperscript{41} They are suggested candidates for
treatment of increased 3-OH-KYN levels in e.g. Huntington’s disease and AIDS dementia.  

### 1.1.4 Kynureninase

Kynureninase (EC 3.7.1.3, KYNase) is a pyridoxal-5’-phosphate (PLP) dependent enzyme. KYNase has an aminotransferase type fold and belongs to family IVa within the aminotransferase superfamily. KYNase catalyzes the β,γ-hydrolytic cleavage of L-KYN and 3-OH-KYN. The properties of the human enzyme have been well characterized. The enzyme exists in homodimeric form. A monomer has a mass of 52.4 kDa. The human enzyme shares 85% homology with the rat variant. The human variant of KYNase can only convert 3-OH-KYN to 3-OH-AA. Rat KYNase is also able to convert KYN to anthranilic acid (AA). It is generally believed that the conversion of KYN to 3-OH-KYN and subsequent hydrolysis to 3-OH-AA is the main route in mammalian brain. Literature is

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**PLP dependent enzymes**

Pyridoxal-5’-phosphate (PLP) dependent enzymes are enzymes that use PLP as their co-enzyme. PLP is the active form of vitamin B6. It is covalently linked to the enzyme by a “Schiff base” linkage with a lysine residue. In most types of enzymes, the exchange of linkage to an amino-group within the substrate initializes the enzymatic reaction. PLP dependent enzymes are divided into at least 5 different fold types.

Aminotransferases form a large distinctive group within the PLP dependent enzymes. They belong to fold types I and IV of PLP dependent enzymes. The group of aminotransferases is further divided into families and subgroups, based on evolutionary and structural relationships.[1-5]

more divergent about the question whether KYNase is able to convert KYN to AA in the periphery and whether this is the preferred route of KYN metabolism in these tissues.\textsuperscript{7, 61-63} In microbial organisms, two distinct enzymes exist for catabolism of KYN and 3-OH-KYN, respectively.\textsuperscript{64}

The mechanism of the KYNase catalytic cycle was studied in detail in \textit{Pseudomonas fluorescens}. The initiating step is formation of an aldimine linkage between KYN and PLP. The key step is the formation of the \textit{gem-diolate} in the presence of water and base. The release of AA shifts the equilibrium towards the product. Finally, pyruvate or alanine and the free co-enzyme are released. The reaction scheme\textsuperscript{65-67} is shown in Figure 3.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Catalytic mechanism of kynureninase\textsuperscript{65-67}}
\end{figure}

The crystal structure of human KYNase reveals, that in addition to binding to PLP, the substrate interacts with a conserved arginine residue. This interaction
induces a conformational change of the enzyme from the open to the closed form of the binding pocket. In this closed state, the substrate is positioned in such a way, that the internal strain within the molecule facilitates formation of the product.\textsuperscript{58} Sigmoidal velocity plots obtained from recombinant human KYNase suggest, that the human enzyme has two binding sites: one catalytic site, and one regulatory non-catalytic site.\textsuperscript{68}

**KYNase inhibitors**

Published KYNase inhibitors can be divided into different classes. The classes and representative inhibitors are shown in Table 2.

<table>
<thead>
<tr>
<th>Table 2 Kynureninase inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compound</strong></td>
</tr>
<tr>
<td>--------------------------------</td>
</tr>
<tr>
<td><strong>Transition state mimics</strong></td>
</tr>
</tbody>
</table>
| dihydro-\textsuperscript{L}-kynurenine | 4-\textit{S} \textit{K}_\textit{i} = 0.3 \textmu M  
4-\textit{R} \textit{K}_\textit{i} = 1.4 \textmu M | Pseudomonas fluorescence | \textsuperscript{69} |
| 2-amino-4-[3'-hydroxyphenyl]-4-hydroxybutanoic acid | \textit{Rat K}_\textit{i} = 0.130 \textmu M  
Human \textit{K}_\textit{i} = 0.100 \textmu M  
Bacteria \textit{K}_\textit{i} = 10 \textmu M | Rat liver kynureninase, recombinant human kynureninase, bacterial kynureninase | \textsuperscript{70} |
| \textit{R}-2-amino-3-(hydroxy-phenyl-phosphinoyl)-propionic acid | \textit{K}_\textit{i} = 4280±100 \textmu M | Pseudomonas fluorescence | \textsuperscript{71} |
| \textit{R}-2-amino-3-(methoxy-phenyl-phosphinoyl)-propionic acid | (\pm) \textit{K}_\textit{i} = 880±50 \textmu M | Pseudomonas fluorescence | \textsuperscript{71} |
| \textit{S}-[2-aminophenyl]-L-cysteine \textit{S,S}-dioxide | \textit{K}_\textit{i} = 0.07 \textmu M | Pseudomonas fluorescence | \textsuperscript{72} |
| \textit{S}-[2-amino-5-methylphenyl]-L-cysteine \textit{S,S}-dioxide | IC\textsubscript{50} = 36 \textmu M | Rat liver kynureninase | \textsuperscript{73} |
|                                 | IC\textsubscript{50} = 11 \textmu M | Rat liver kynureninase | \textsuperscript{73} |
Table 2 (continued)

<table>
<thead>
<tr>
<th>Substrate analogs</th>
<th>Bacteria $K_i = 10 \mu M$</th>
<th>Rat $K_i = 175 \mu M$</th>
<th>Human $K_i = 15 \mu M$</th>
<th>Partly purified enzymes</th>
<th>68</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-methoxybenzoylalanine (mMBA)</td>
<td>$IC_{50} = 4.65 \mu M$</td>
<td>Rat brain homogenate</td>
<td>47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-methoxybenzoylalanine (oMBA)</td>
<td>$IC_{50} = 3\pm0.8 \mu M$</td>
<td>Rat liver homogenate</td>
<td>46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>naphthylbenzoylalanine</td>
<td>Bacteria $K_i = 5 \mu M$</td>
<td>Human $K_i = 22 \mu M$</td>
<td>Pseudomonas fluorescens, recombinant human</td>
<td>74</td>
<td></td>
</tr>
</tbody>
</table>

**Mechanism based inhibitors**

| S-(o-nitrophenyl)-L-cysteine | $K_i = 100 \mu M$ | Pseudomonas marginalis | 75 |
| β-Cl-L-alanine | $K_i = 8000 \mu M$ | Pseudomonas marginalis | 75 |
| oestron sulphate | $K_i = 82\pm6 \mu M$ | Partially purified rat liver enzyme | 76 |

An important class of KYNase inhibitors consists of *transition state mimics*. Compounds of this class have a tetrahedral moiety, which resembles the gem-diolate intermediate (Figure 3). The most active of these series are the *S*-aryl-*L*-cysteine *S,S*-dioxides.\textsuperscript{72, 73} The second group of compounds consists of *substrate analogs*. Among those, *o*-methoxybenzoylalanine (oMBA) is the most studied compound. Similar to, but less pronounced than the KMO inhibitor mNBA, oMBA elevated extracellular KYNA levels in rat brain. Sedative and anticonvulsive properties were seen.\textsuperscript{46, 47}

*Mechanism based inhibitors* are designed to interfere with the catalytic cycle of the enzyme. A group of β-substituted amino acids was tested. The active
compounds eliminate their \( \beta \)-substituent, releasing aminoacrylate compounds that most probably react with active site nucleophilic groups and thus inactivate the enzyme. These compounds are not KYNase selective.\(^75\) Oestrone sulphate was found to inhibit KYNase competitively, causing abnormal tryptophan metabolism in women using this compound as oral contraceptive or menopausal hormone.\(^76\)

### 1.1.5 Kynurenine aminotransferase

Kynurenine aminotransferase (KAT, EC 2.6.1.7) is the enzyme that converts key intermediate kynurenine into kynurenic acid. KAT is a PLP-dependent enzyme. It belongs to subgroup 1\(^{57}\) or 1b\(^{27}\) of the PLP-dependent aminotransferases. KAT is a functional homodimer, with two catalytic sites at the dimeric interface. The catalytic cycle, shown in Figure 4, requires the co-enzyme PLP, the substrate KYN and an \( \alpha \)-ketoacid (co-substrate).

**Figure 4**

![KAT catalytic cycle](image)

Initially, PLP is bound to a conserved lysine residue in the active site by an aldimine linkage. When kynurenine binds to the binding site, the bond between the co-enzyme and the lysine residue is broken, and the co-enzyme binds to the \( \alpha \)-amino group of the substrate. This \( \alpha \)-amino group is transferred to the co-enzyme, leaving the \( \alpha \)-keto acid analog of KYN as the enzymatic product.
Spontaneous ring closure of the keto acid affords kynurenic acid. The PLP form is regenerated from the pyridoxamine phosphate (PMP) form by transfer of the amino-group to the α-ketoacid. Studies on crystal structures indicate, that the binding site can accommodate the substrate and a co-substrate of the size of pyruvate simultaneously. Whether the substrate and co-substrate are bound simultaneously or in turns during the catalytic cycle remains to be elucidated.

Four KAT isoforms have been published (KAT I-IV). KAT I and KAT II are most studied.

*KAT I* is identical to glutamine transaminase K (EC 2.6.1.64) and cysteine S-conjugate β-lyase (EC 4.4.1.13). Human KAT I has 422 amino acid residues per monomer. The rat enzyme exists as a mitochondrial and a soluble, cytosolic form. The mitochondrial form appears mostly in brain, the cytosolic form in the periphery. Differences between the mitochondrial and cytosolic forms originate in different splicing of the same gene. This results in the presence of an additional peptide (32 amino acids) in the mitochondrial form, which leads the enzyme to the mitochondrial matrix. In humans, only the cytosolic form has been identified. In the brain, KAT I is primarily located in glia, but is also found in neurons. KAT I has a pH optimum at pH 9.0-9.5. However, studies in human KAT I showed, that KAT I is active at a broader pH range, but is inhibited by Tris buffer at physiological pH ranges. KAT I prefers pyruvate as the co-substrate. Interestingly, recombinant human KAT I prefers several other 2-oxoacids.
especially $\alpha$-ketobutyrate, as the amino group acceptor over pyruvate. KAT I transaminates most naturally occurring, preferably hydrophobic, amino acids, but is inhibited by Gln, Trp, Phe, and Cys.\(^{88,89}\)

The human KAT I enzyme shares 82\% similarity with the rat enzyme.\(^{89}\) Crystal structures of the human PLP-bound, the PMP bound and the PLP- and inhibitor-bound KAT I are available.\(^{79,80}\) A missense mutation in KAT I was found in spontaneously hypertensive rats.\(^{90}\)

\textit{KAT II} is the second isoform and shows $L$-aminoacidipe aminotransferase (EC 2.6.1.39) activity.\(^{91}\) The enzyme has 425 amino acid residues per monomer. The preferred co-substrate is $2$-oxoglutarate, and enzymatic activity has a pH optimum at 7.0-7.5. KAT II is thought to be responsible for ca. 75\% of KYNA production in mammalian brain.\(^{88}\) Rat KAT II specific antibodies have been developed. These antibodies showed, that rat KAT II is primarily localized in astrocytes.\(^{92}\) Having broad substrate specificity, KAT II catalyzes the transamination of KYN, $3$-OH-KYN, Trp, Phe, Asn, Asp and various other amino acids. As co-susbtrates, a variety of $\alpha$-ketoacids can be accommodated.\(^{88,93}\) Catalytic efficiency of human KAT II is highest for aminoacidipe and KYN. Although the $K_m$ for KYN is rather high, the very high turnover rate ($k_{cat}$) indicates that conversion of KYN to KYNA is one of the major physiological functions of the enzyme.\(^{93}\) Recently, crystal structures of human KAT II in complex with PLP with and without KYN and with $\alpha$-ketoglutarate have become available.\(^{93-95}\) To study the function of KAT II, knock out ($kat^{-2/-}$) mice were generated. Interestingly, brain tissue KYNA levels were reduced only in the first postnatal days in these mice. At this age, mice showed neuronal (enlarged dendritic spines, increased spine density) and behavioral (hyperactivity, motor coordination) abnormalities, and enhanced vulnerability to QUIN induced lesions. In adult animals, KYNA levels in brain tissue were normalized, probably due to compensation by other KAT isoforms. In liver tissue, KYNA was reduced by $>90\%$ throughout the lifecycle, but caused no apparent abnormalities.\(^{52,96}\)

\textit{KAT III} and \textit{KAT IV} are less established isoforms of KAT. KAT III was identified from human and mouse cDNA libraries. It is expressed most abundantly in peripheral tissues.\(^{97}\) Biochemical characterization and crystal structures of mouse KAT III showed close resemblance to KAT I. As KAT I, mouse KAT III shows relatively broad substrate and co-substrate specificity. In contrast to KAT I however, mouse KAT III prefers hydrophilic amino acids. The enzyme has a pH-optimum at pH 9-10 and is most active at relatively high temperatures (50 –
60°C). KAT I and III are thought to compensate for KAT II activity in kat-2^-/- mice.98

**Glutamate receptors**

Glutamate receptors can be divided into metabotropic and ionotropic glutamate receptors (mGluRs and iGluRs). Metabotropic glutamate receptors are G-protein coupled receptors and account for long-term effects of glutamatergic neuromodulation. Ionotropic glutamate receptors are ligand operated ion channels. iGluRs consist of heterogeneous subunits, determining activity and sensitivity of the channel.

Three types of iGluRs are known: NMDA, AMPA and kainate receptors. NMDA (N-methyl-D-aspartate, depicted) receptors are assemblies of subunits NR1, NR2 (NR2A-D) and NR3 (NR3A-B). Activity of the NMDA receptor is regulated at the agonist site and a variety of modulatory sites. The most important modulatory site is the allosteric glycine site. This site is important in regulation of open-time and desensitization rate. Other sites are the Zn²⁺ site, the Mg²⁺ site and the PCP (phencyclidine) site. PCP, as well as ketamine and MK-801 can induce symptoms that closely resemble symptoms seen in schizophrenic patients.

KAT IV is identical to mitochondrial aspartate aminotransferase (EC 2.6.1.1). The enzyme has a pH optimum of 8. It is mostly present in neurons, but is also found in astrocytes.99

The relative percentages of KAT I, KAT II and KAT IV isoform activities were determined in mouse, rat, and human brain. In mouse, KAT IV is the major isoform (63.0%), whereas in rat and human, KAT II is most abundant (54.1% and 58.7%, respectively). Mitochondrial localization of KAT I, KAT IV and also of KMO might be linked to major impact of mitochondrial electron transfer chain inhibitors on the kynurenine pathway.99 Further relevance of KAT I, III and IV under physiological and pathological circumstances remains to be elucidated.

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1.1.6 Kynurenic acid

Kynurenic acid (KYNA) is the product of KYN transamination by KATs. KYNA has distinctive physiological functions. It is an antagonist at the strychnine insensitive glycine coagonist site of NMDA receptors with an IC\textsubscript{50} of 8-15 µM in the absence of glycine.\textsuperscript{100-102} At α7* nicotinic acetylcholine receptors (nAChRs), KYNA shows non-competitive antagonism with an IC\textsubscript{50} of 7 µM.\textsuperscript{102} At much higher, non-physiological millimolar concentrations, KYNA is an antagonist at all ionotropic glutamate receptors.\textsuperscript{103} A biphasic effect of KYNA on AMPA receptors was observed in hippocampal cell layers. At low concentrations (10 µM), the effect of AMPA is potentiated. At higher concentrations, the expected competitive antagonism is observed. Potentiation is thought to be mediated through positive allosteric modulation, but needs to be further established.\textsuperscript{104} Recently, KYNA was found to activate GPR35 orphan receptors at micromolar concentrations (EC\textsubscript{50} = 39.2 µM for human GPR35).\textsuperscript{105}

KYNA is present in the brain and in peripheral tissues.\textsuperscript{106, 107} In the brain, KYNA is thought to have important physiological functions. As will be discussed in section 1.1.7, KYNA levels are highly regulated and levels vary over the specific brain regions. In rats, estimated levels of KYNA are 17 nM (with an expected 5-10 fold underestimation) in striatal ECF and 138 nM in plasma.\textsuperscript{108} In human cerebrospinal fluid, KYNA levels of 0.97 nM\textsuperscript{3} and 1.06 nM\textsuperscript{4} were reported. KYNA production in the periphery is less well investigated. Kidneys, liver, heart and vascular endothelium synthesize KYNA and KYNA is present in urine, serum, amniotic fluid, and in the retina.\textsuperscript{109, 110} In the gastrointestinal tract, KYNA concentrations can be up to micromolar concentrations (highest in distal ileum: 16 µM). Preferential localization of GPR35 in the gastrointestinal tract suggests a physiological role of KYNA acting on these receptors in this area.\textsuperscript{111}

1.1.7 Regulation of KYNA levels

KYNA levels are regulated by an array of different mechanisms. Many of these mechanisms might function as targets for pharmacological manipulation of KYNA levels.

1.1.7.1 Transporters

KYNA production depends on the availability of its direct precursor, KYN. The transport of KYN towards KAT enzymes within astrocytes depends on the sodium independent large neutral amino acid transporter.\textsuperscript{112} Neuronal uptake occurs by a sodium dependent mechanism, which is less efficient.\textsuperscript{113} Transport
mechanisms in general depend on ionic environment and availability of glucose.\textsuperscript{114, 115}

Exogenous and endogenous compounds can modify the transport of KYN. Homocysteine is transformed \textit{in vivo} to S-adenosylhomocysteine in mammalian brain and aortic rings\textsuperscript{109, 116} or cysteine in mosquito\textsuperscript{117}. Both metabolites are suggested endogenous augmenters of KYN transportation. KYN transportation and KYNA synthesis are dose-dependently inhibited by L-leucine and L-phenylalanine in human cultured astrocytes by competition of the amino acids at the KYN transporter site.\textsuperscript{118} Similarly, the observed reduction of KYNA synthesis by L-\(\alpha\)-amino adipic acid can be explained by impaired mitochondrial KYN uptake.\textsuperscript{119}

Transport of compounds that influence KYNA synthesis on other than KYN transport levels are of importance in at least some cases. The mGluR agonists quisqualate, \(L\)-(+)\-2-amino-4-phosphonobutyric acid (L-AP4), 4-carboxy-3-hydroxyphenylglycine (4C3HPG) and L-serine-O-phosphate (L-SOP) are relatively weak KAT II inhibitors. Interestingly, the compounds were found to have much lower apparent IC\(_{50}\) values in tissue slices than for isolated KAT II enzymes. The compounds are actively transported into cells over a Na\(^+\)-dependent transporter. Therefore, although the real IC\(_{50}\) values are rather high, concentrations high enough to effectively inhibit KAT II activity might be reached within these cells by this active transport.\textsuperscript{114, 120, 121} The same holds for L-\(\alpha\)-amino adipic acid, which is transported into astrocytes by high affinity Na\(^+\) independent uptake mechanisms.\textsuperscript{114} Accordingly, the action of quisqualate and L-\(\alpha\)-amino adipic acid was shown to be dependent on the developmental status of the transporters involved.\textsuperscript{114}

\textit{1.1.7.2 Ionic concentrations, cell energy status}

KYNA synthesis depends on ionic concentrations in the KAT surrounding environment. Na\(^+\) dependent transport mechanisms are of direct influence on KYN availability within cells. Depolarizing conditions (veratridine, high K\(^+\)) decrease extracellular KYNA concentrations.\textsuperscript{114} Impaired energy metabolism influences KYNA synthesis. Glucose deprivation, impaired glucose uptake, and inhibition of glycolysis attenuate KYNA synthesis. This is at least partly caused by impaired cellular KYN uptake, and can be reversed by glycolytic or tricarboxylic acid cycle intermediates.\textsuperscript{120, 122} In terms of energy status, mitochondrial complex inhibitors have been studied. Mitochondrial complex inhibitors MPP\(^+\) (complex I)\textsuperscript{123, 124}, 3-nitropropionic acid
(complex II)\textsuperscript{123, 125} and sodium azide (complex IV)\textsuperscript{125} decrease KYNA availability. The main mechanism behind this observation seems to be KAT depletion in affected cells.\textsuperscript{124, 125} Finally, ammonium acetate was shown to inhibit KYNA synthesis \textit{in vitro} and \textit{in vivo} in rats by suggested metabolic dearrangements in astrocytes.\textsuperscript{126}

\subsection*{1.1.7.3 KAT activity – pH, co-enzyme, co-substrates}

The activity of KAT enzymes is pH dependent. Therefore, the pH in the surrounding environment is of importance for the rate of KYNA synthesis.\textsuperscript{88, 89} KAT activity depends on availability of the co-enzyme pyridoxal 5\textsuperscript{-}phosphate (PLP). The anti-tuberculosis drug isonicotinic acid hydrazide was shown to significantly decrease liver KAT activity in rats. This resulted in decreased urinary excretion of KYNA. A suggested explanation is reaction of isonicotinic acid hydrazide with the co-enzyme, resulting in reduced availability of the co-enzyme. The precise nature of the reaction was not specified.\textsuperscript{127}

The effect of availability of $\alpha$-ketoacids, which function as co-substrates, on KAT activity is more complex. In general, broad co-substrate specificity is observed for KATs.\textsuperscript{89, 115, 117} Good availability of $\alpha$-ketoacids enhances KAT activity in rat brain, but to varying extents. This results in directly enhanced brain KYNA concentrations. Recombinant mosquito KAT enzymes were found to be inhibited dose-dependently by most studied $\alpha$-ketoacids at higher concentrations.\textsuperscript{117} No such effect was seen in rat tissue.\textsuperscript{115, 117}

Apart from its direct involvement in KAT activity as a co-substrate, the $\alpha$-ketoacid pyruvate can influence metabolic activities within cells. It is the end-product of glycolysis and a direct substrate of the tricarboxylic acid cycle. In this way, it can restore cell metabolism in energy deprived circumstances.\textsuperscript{115, 122}

\subsection*{1.1.7.4 Competing reactions}

The balance between KYN metabolism into 3-OH-KYN, AA and KYNA is tightly regulated. Inhibition of one or more of the KYN metabolizing enzymes results in a shift in this balance. In particular, inhibitors of KMO and KYNase are commonly known tools to pharmacologically elevate KYNA levels (see sections 1.1.3 and 1.1.4).

\subsection*{1.1.7.5 Direct KAT inhibition}

Although the list of compounds that have been found to inhibit KAT is relatively extensive, until recently, no KAT inhibitor was known that was able to decrease
brain KYNA levels \textit{in vivo per se}. This results mainly from a lack of selectivity and specificity.

As for KMO and KYNase inhibitors, KAT substrate KYN was used as the template for the design of potential KAT inhibitors. Examples of KYN analogs that were studied for KAT inhibitory activity are shown in Table 3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\text{IC}_{50}$ or % KYNA production from control</th>
<th>Species/method</th>
<th>Mechanism</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{D,L-5-bromokynurenine}$</td>
<td>micromolar</td>
<td>\textit{in vitro}</td>
<td>selective KAT II over KAT I</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>48% @ 100 $\mu$M</td>
<td>rat brain slices</td>
<td>KAT inhibition</td>
<td>128</td>
</tr>
<tr>
<td>$\text{L-5-chlorokynurenine}$</td>
<td>39% @ 100 $\mu$M</td>
<td>rat brain homogenate</td>
<td>KAT inhibition</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>14 $\mu$M</td>
<td>rat brain homogenate</td>
<td>KAT inhibition</td>
<td>129</td>
</tr>
<tr>
<td>$\text{S-4-(ethylsulfonyl)-benzoylalanine}$</td>
<td>71 $\mu$M</td>
<td>rat liver homogenate</td>
<td>KAT II inhibition</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>6.1 $\mu$M</td>
<td>partially purified KAT II (rat liver)</td>
<td>KAT II inhibition</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>36% @ 1 mM</td>
<td>recombinant human KAT II</td>
<td>KAT II inhibition</td>
<td>130</td>
</tr>
</tbody>
</table>

A close analog of KYN, 5-chlorokynurenine (5-Cl-KYN) was published as a potent inhibitor of KAT enzymes. However, due to a lack of KAT selectivity, this compound is not able to lower KYNA levels \textit{in vivo} [personal communication]. Interestingly, a structural analog of 5-Cl-KYN, 4-chlorokynurenine, is a prodrug of 7-chlorokynurenic acid (7-Cl-KYNA). 7-Cl-KYNA is a prototypical antagonist of the glycine coagonist site at NMDA receptors.\textsuperscript{131, 132} \textit{S-4-(Ethylsulfonyl)-benzoylalanine} (S-ESBA) is the only known KYN analog that selectively inhibits KAT II. In a microdialysis study in rats, local infusion of S-ESBA (5 mM, 2 hours) was able to decrease KYNA levels in hippocampus.\textsuperscript{129} Unexpectedly, the inhibitory potency of S-ESBA was 10-20 fold less for human KAT II.\textsuperscript{129, 130} A second group compounds tested for their KAT inhibitory activity comprises naturally occurring amino acids and their derivatives (Table 4). Along with the third group, the cysteine analogs (Table 5), this group of compounds is of particular interest regarding their roles in physiological and pathological circumstances. Finally, some (semi)-synthetic compounds are listed in Table 6.
### Table 4 KAT inhibitors: Naturally occurring amino acids and derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ or % KYNA production from control</th>
<th>Species/method</th>
<th>Mechanism</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-aspartate</td>
<td>300/720/73 µM</td>
<td>rat: cortical slices/KAT I in brain homogenate/KAT II in brain homogenate</td>
<td>KAT inhibition</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>58.9% @ 0.1 mM</td>
<td>bovine aortic endothelial cells</td>
<td>KAT inhibition</td>
<td>110</td>
</tr>
<tr>
<td>L-glutamate</td>
<td>790/1300/790 µM</td>
<td>rat: cortical slices/KAT I in brain homogenate/KAT II in brain homogenate</td>
<td>KAT inhibition</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>72.0% @ 0.1 mM</td>
<td>bovine aortic endothelial cells</td>
<td>KAT inhibition</td>
<td>110</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>2 mM: pH 7.0: 78% pH 8.0: 43% pH 9.5: 3%</td>
<td>brain homogenate</td>
<td>KAT I inhibition</td>
<td>88</td>
</tr>
<tr>
<td>L-methionine</td>
<td>1930 µM /397 µM/ no inhib</td>
<td>rat: cortical slices/KAT I in cortical homogenate/ KAT II in cortical homogenate</td>
<td>KAT I inhibition</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>68.4% @ 500 µM</td>
<td>rat aortic rings</td>
<td>KAT inhibition</td>
<td>117</td>
</tr>
<tr>
<td>N(y)-Nitro-L-arginine</td>
<td>341 µM</td>
<td>KAT I in rat brain homogenate</td>
<td>KAT I inhibition</td>
<td>134</td>
</tr>
<tr>
<td>N(y)-nitro-L-arginine methyl ester</td>
<td>421 µM</td>
<td>KAT I in rat brain homogenate</td>
<td>KAT I inhibition</td>
<td>134</td>
</tr>
</tbody>
</table>

### Table 5 KAT inhibitors: Cysteine analogs

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ or % KYNA production from control</th>
<th>Species/method</th>
<th>Mechanism</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-cysteine</td>
<td>1400 µM/no inhib/3390 µM</td>
<td>rat cortical slices/KAT I in rat cortical homogenate/KAT II in rat cortical homogenate</td>
<td>KAT II inhibition</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>70.6 µM</td>
<td>rat aortic rings</td>
<td>KAT inhibition</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>enhancement @ 0.31-2.5 mM, inhib @ 5-40 mM</td>
<td>recombinant AeKAT (mosquito)</td>
<td>inhibition: competitive / covalent KAT inhibition</td>
<td>117</td>
</tr>
<tr>
<td>L-cysteine sulphinate</td>
<td>80 µM/no inhib/2 µM</td>
<td>rat cortical slices/KAT I in rat brain homogenate/KAT II in rat brain homogenate</td>
<td>KAT inhibition</td>
<td>133</td>
</tr>
<tr>
<td>L-cysteate</td>
<td>1770/5380/1550 µM</td>
<td>rat cortical slices/KAT I in rat brain homogenate/KAT II in rat brain homogenate</td>
<td>KAT inhibition</td>
<td>133</td>
</tr>
</tbody>
</table>
### Table 5 (continued)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration/Effect</th>
<th>Experimental System</th>
<th>KAT Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-homocysteine sulphinate</td>
<td>2900 µM/no inhib/1590 µM</td>
<td>rat cortical slices/KAT I in rat brain homogenate/KAT II in rat brain homogenate</td>
<td>KAT inhibition</td>
</tr>
<tr>
<td>L-homocysteate</td>
<td>3930/5000/2100 µM</td>
<td>rat cortical slices/KAT I in rat brain homogenate/KAT II in rat brain homogenate</td>
<td>KAT inhibition</td>
</tr>
<tr>
<td></td>
<td>enhancement @ 100-500 µM/IC50 6400 µM</td>
<td>rat cortical slices</td>
<td>KAT inhibition</td>
</tr>
<tr>
<td>D,L-homocysteine</td>
<td>566 µM/8046 µM</td>
<td>KAT I/KAT II in rat cortical homogenate</td>
<td>KAT inhibition</td>
</tr>
<tr>
<td></td>
<td>@&lt;100 µM: increase KYNA, @500uM: 61.1% KYNA prod</td>
<td>rat aortic rings</td>
<td>KAT inhibition, conversion to adenosyl-homocysteine</td>
</tr>
<tr>
<td></td>
<td>54 µM</td>
<td>bovine aortic endothelial cells</td>
<td>KAT inhibition</td>
</tr>
<tr>
<td>S-adenosylhomocysteine</td>
<td>enhancement @ 30-80 µM/63.4% @ 1000 µM</td>
<td>rat cortical slices</td>
<td>KAT II inhibition</td>
</tr>
<tr>
<td></td>
<td>no inhib/260 µM</td>
<td>KAT I/KAT II in rat cortical homogenate</td>
<td>KAT inhibition</td>
</tr>
<tr>
<td></td>
<td>@&lt;80 µM enhanced to 124%, @500 µM: decreased to 62%</td>
<td>rat aortic rings</td>
<td>KAT inhibition</td>
</tr>
<tr>
<td>dichlorovinyl-cysteine</td>
<td>48.6% @ 50 µM</td>
<td>rat cortical tissue slices</td>
<td>non-specific KAT inhibitor</td>
</tr>
</tbody>
</table>

*Note: KYNA = kynurenic acid, IC50 = inhibitory concentration.*
Table 6 KAT inhibitors: Other compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \text{IC}_{50} ) or % KYNA production from control</th>
<th>Species/method</th>
<th>Mechanism</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-( \alpha )-aminoadipate</td>
<td>75 and 53% @ 500 ( \mu )M KYN, 300 and 500 ( \mu )M aminoadipate</td>
<td>rat microdialysis</td>
<td>allosteric inhibition</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>micromolar</td>
<td>\textit{in vitro}</td>
<td>selective KAT II over KAT I inhibition</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>10-30 ( \mu )M</td>
<td>rat cortical slices</td>
<td>KAT II inhibition</td>
<td>114</td>
</tr>
<tr>
<td>Quisqualate</td>
<td>micromolar</td>
<td>\textit{in vitro}</td>
<td>selective KAT II over KAT I inhibition</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>520 ( \mu )M</td>
<td>partially purified KAT II</td>
<td>KAT II inhibition</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>1-100 ( \mu )M / 10-1000 ( \mu )M</td>
<td>rat cortical tissue slices/rat brain homogenate</td>
<td>KAT II inhibition, active transport</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>15.9 ( \mu )M</td>
<td>rat cortical slices</td>
<td>KAT II inhibition</td>
<td>114</td>
</tr>
<tr>
<td>4-carboxy-3-hydroxy-phenylglycine (4C3HPG)</td>
<td>10-10000 ( \mu )M / 65%act @ 200 ( \mu )M</td>
<td>rat cortical tissue slices/rat brain homogenate</td>
<td>KAT II inhibition, active transport</td>
<td>120</td>
</tr>
<tr>
<td>L-2-amino-4-phosphono-butric acid (L-AP4)</td>
<td>10-10000 ( \mu )M / 100-10000 ( \mu )M</td>
<td>rat cortical tissue slices/rat brain homogenate</td>
<td>KAT II inhibition, active transport</td>
<td>120</td>
</tr>
<tr>
<td>L-serine-O-phosphate (L-SOP)</td>
<td>100-10000 ( \mu )M / 10000-100000 ( \mu )M</td>
<td>rat cortical tissue slices/rat brain homogenate</td>
<td>KAT II inhibition, active transport</td>
<td>120</td>
</tr>
<tr>
<td>Aminoxyacetic acid</td>
<td>0.1-1 ( \mu )mol</td>
<td>\textit{in vivo}, rat striatum</td>
<td>non-specific transaminase inhibitor</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>25 ( \mu )M</td>
<td></td>
<td>non-specific transaminase inhibitor</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>3.20 ( \mu )M</td>
<td>bovine aortic endothelial cells</td>
<td>KAT inhibition</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>35.5% @ 50 ( \mu )M</td>
<td>rat cortical tissue slices</td>
<td>non-specific KAT inhibitor</td>
<td>115</td>
</tr>
<tr>
<td>( \gamma )-acetylenic GABA</td>
<td>43 ( \mu )M /750 ( \mu )M</td>
<td>rat hippocampal homogenate/slices</td>
<td>non-specific KAT inhibitor</td>
<td>137</td>
</tr>
</tbody>
</table>
Table 6 (continued)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Tissue Type</th>
<th>Effect</th>
<th>inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>trans-pyrorline-2,4-dicarboxylate (trans-PDC)</td>
<td>80% @ 1 mM</td>
<td>rat brain homogenate</td>
<td>KAT II inhibition</td>
<td>120</td>
</tr>
<tr>
<td>Veratridine</td>
<td>3-5 µM</td>
<td>rat cortical slices</td>
<td>disturbance of energy</td>
<td>114</td>
</tr>
<tr>
<td>BFF 122</td>
<td>7% @ 1 mM</td>
<td>rat brain homogenate</td>
<td>KAT II inhibition</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>10% @ 100 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30% @ 10 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0% @ 1 mM</td>
<td>partially purified KAT II</td>
<td>KAT II inhibition</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>0% @ 100 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0% @ 10 µM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0% @ 1 µM</td>
<td></td>
<td></td>
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The compounds in Table 6 are occasionally used as research tools. Recently, BFF 122 was published as a KAT II selective inhibitor. The compound was used as a research tool to investigate kynurenine metabolism in brain by local application. The compound is not able to cross the blood brain barrier [personal communication].

1.1.7.6 Mechanism based inhibitor: Vinylglycine

Pyridoxal 5'-phosphate dependent enzymes can be inhibited by mechanism based inhibitors. These compounds initially bind with relatively low affinity. Catalytic processing by the enzyme results in a reactive intermediate, which binds covalently to an amino acid side chain in the active site of the enzyme. The covalently bound compound irreversibly inhibits the enzyme. For KATs, this was shown with vinylglycine. Apart from being converted into α-ketobutyrate, this compound showed irreversible inhibition of yeast KAT by a γ-addition reaction.

1.1.7.7 Influx and efflux of KYNA

KYNA cannot cross the blood brain barrier by passive means. A probenecid sensitive carrier can transport KYNA from the brain into the periphery. Administration of probenecid increases KYNA levels in brain by competing with the efflux carrier. Probenecid in combination with systemic KYN administration increases levels of KYNA in brain.
Systemic KYNA administration does not raise KYNA levels in brain. In rats that were treated with glucosamine-KYNA in combination with probenecid in the periphery, a KYNA-related reduction of population spike amplitudes in the hippocampus was observed already at low doses of the drug. Administration of KYNA had no electrophysiological effect. Presumably, glucosamine-KYNA is more easily transported into the brain and is hydrolyzed locally to KYNA.\textsuperscript{140}

One of the explanations of the decrease of KYNA concentrations following \(\alpha\)-aminoacidipic acid administration is interference with efflux of the compound from astrocytes. Although this has not been proven, interference of some compounds with transport mechanisms at the cellular level might be considerable.\textsuperscript{119}

There are no KYNA metabolizing enzymes known. The only way of systemic KYNA elimination is by renal excretion.\textsuperscript{106}

\textbf{1.1.7.8 Neurotransmitters}

\(D\text{-}amphetamine\textsuperscript{141}\), selective \(D_1\) and \(D_2\) agonists\textsuperscript{142} and \(L\text{-}DOPA\textsuperscript{143}\) cause brain specific decreases in KYNA levels, as determined in whole brain tissue and by microdialysis. The mechanisms of KYNA reduction induced by dopaminergic signaling are not completely clear, but most probably involve direct dopaminergic signaling of \(D_1\) and \(D_2\) receptors on astrocytes. Decreases in KYNA were especially pronounced in young rats and could be antagonized by \(D_1\)- and \(D_2\)-selective antagonists. Interestingly, whereas aselective or \(D_2\)-selective antagonists alone did not change KYNA levels in these experiments, a \(D_1\)-selective antagonist increased KYNA levels. These effects of dopamine agonists on KYNA directly enhance neuronal vulnerability, which could be reverted by administration of KMO inhibitor Ro 61-8084.\textsuperscript{141-144}

Nicotine has a biphasic effect on KYNA brain tissue levels in hippocampus, striatum and cortex and extracellular levels in hippocampus, as determined in rats.\textsuperscript{145} After 4-6 days of nicotine administration, decreased KYNA levels were seen. After 10-15 days, KYNA levels were increased. Mechanisms could involve desensitization and subsequent supersensitization of nAChRs. These nAChRs regulate extracellular levels dopamine, which in turn regulate KYNA availability. An additional possibility involves altered Glu transmission. Presynaptic nAChRs on glutamate terminals regulate extracellular glutamate levels. Glutamate was shown to decrease extracellular KYNA levels, probably through direct effects on glial cells.\textsuperscript{146} Therefore, nicotine, acting on the presynaptic nAChRs, might alter Glu transmission towards glia by causing desensitization and supersensitization of these nAChRs.\textsuperscript{102,145}
1.1.7.9 Pharmacological compounds that influence KYNA levels

Some compounds used in research or clinic have pronounced effects on the kyurenine pathway and KYNA levels in vitro or in vivo. NO-donors S-nitroso-N-acetylpenicillamine (SNAP) and 3-morpholinosydnonimine (SIN-1) were shown to increase KYNA production in rat cortical slices. The compounds had no effect on KAT activity. Attenuation of the effects by the antioxidant L-ascorbate suggests that the effect is mediated through reactive oxygen species.\textsuperscript{147} FK506, a modulator of intracellular Ca\textsuperscript{2+} levels, increases KYNA production in rat cortical slices. The mechanism is unknown, but KAT independent.\textsuperscript{148} The anticonvulsant carbamazepine activates KAT I, but not KAT II, resulting in increased KYNA production in rat cortical slices.\textsuperscript{149} NSAIDs have effects on KYNA concentrations in vivo. A selectivity and COX-1 selective inhibitors elevate rat KYNA levels in brain tissue. COX-2 selective inhibitors decrease KYNA levels in brain tissue. Both effects seem to be mediated through prostaglandins E1/E2.\textsuperscript{150, 151} After chronic (1 month – 1 year) treatment of rats with aselective dopamine antagonist haloperidol, D\textsubscript{2} antagonist raclopride or atypical antipsychotic clozapine, decreased brain tissue and extracellular KYNA levels were seen. These effects were not observed after acute or subchronic (1 week) treatment. Therefore, most probably, other mechanisms than direct action on dopamine receptors are involved. Since KYNA synthesis was decreased selectively, the proposed mechanism behind this observation is that KAT activity is changed by altered cell energy metabolism.\textsuperscript{152}

1.1.8 KYNA physiology

Physiological implications of the presence of KYNA are being studied extensively. Different levels of regulation through KYNA can be distinguished. The role of KYNA is concentration and brain region specific. At the neuronal level, the exact locations of receptors on neurons, as well as the nature of the affected neurons are of importance. Most research of the effects of KYNA on specific neurotransmitter systems focuses on cholinergic, glutamatergic and dopaminergic systems. Some examples will be given of the results of research focusing on the effects of KYNA on physiological functioning.

1.1.8.1 Regulation at the neuronal level: KYNA effects on neuronal activity

A set of electrophysiological studies proved the physiological effects of KYNA via \(\alpha7\) nAChRs. First, it was demonstrated that KYNA decreases current amplitudes in hippocampal neurons that are activated by \(\alpha7\) nAChRs. Subsequently, the
effect of KYNA via interneurons was investigated. Activation of \( \alpha 7^\ast \) nAChRs on GABAergic hippocampal interneurons induces a current in postsynaptic neurons. The amplitude of this postsynaptic current, again, can be reduced by KYNA.\textsuperscript{102} Currents in hippocampal interneurons are mainly caused by glutamatergic transmission. Blockade of this transmission results in a residual current, caused by cholinergic synaptic transmission. This is the residual current that is blocked by KYNA acting on \( \alpha 7^\ast \) nAChRs.\textsuperscript{153} A decrease of amplitudes is seen only at higher KYNA concentrations. A recent report shows, that at nanomolar concentrations, a slight increase in amplitudes is seen.\textsuperscript{154} These examples emphasize, that KYNA might be involved in different levels of regulation of neuronal activity in the brain.

1.1.8.2 Region specific KYNA effects: KYNA regulated acetylcholine release

Cortical cholinergic systems are innervated by GABAergic neurons from the nucleus accumbens. Local infusion of high concentrations of KYNA in the nucleus accumbens induced ACh efflux in the cortex, as determined by microdialysis. These effects could be reproduced by AMPA/kainate and NMDA antagonists, suggesting that effects were mediated by aspecific inhibition of ionotropic glutamate receptors.\textsuperscript{155} In rat cortex, extracellular KYNA levels were decreased by local infusion of KAT II inhibitor S-ESBA. This resulted in an elevation of cortical ACh levels. The effect of S-ESBA could be blocked by co-infusion of KYNA. Accordingly, systemically administered \( D \)-amphetamine results in enhanced ACh release in the cortex, which could be attenuated by cortical KYNA infusion or systemic KYN administration. The mechanisms of local regulation of Ach release are thought to be mediated by aspecific iGlu inhibition or indirectly via glutamate and dopamine mediated cascades.\textsuperscript{156, 157} These opposite effects of KYNA in the nucleus accumbens and cortex on cortical ACh release exemplify the region specific effects of KYNA on neuronal systems.

1.1.8.3 KYNA effects on glutamate, norepinephrine and dopamine

As discussed previously, KYNA concentrations are influenced by dopaminergic and glutamatergic systems. Inversely, KYNA has effect on release of glutamate, norepinephrine and dopamine in the brain. The effect of KYNA on glutamate release was studied in more detail in synaptosomes. In synaptosomes, release of Glu or Asp can be invoked by nicotine or 5-hydroxyindole, a positive modulator of \( \alpha 7^\ast \) nAChRs. KYNA was able
Depolarization induced release of glutamate or [³H]-D-Asp from synaptosomes was also inhibited by KYNA. The blockade was not mediated through selective antagonism of α7* nAChRs. Involvement of NMDA receptors was also ruled out. In microdialysis studies, KMO inhibitors were able to increase KYNA concentrations in cortex, caudate and hippocampus. Surprisingly, Glu concentrations were decreased in the caudate region only, and were unaltered in hippocampus and cortex. Decrease of Glu concentrations in the caudate could be reproduced by a selective α7* nAChR antagonist. This suggests, that KYNA mediated Glu regulation is brain region specific and is mediated, at least partly, through α7* nAChRs.

The activity of the noradrenergic locus coeruleus is regulated by excitatory and inhibitory neurotransmitters. Electrophysiology studies showed, that nicotine has a stimulatory effect on the locus coeruleus, which can be divided into an initial short-lasting phase and a subsequent, more moderate, long-lasting phase. The short-lasting phase is thought to be mediated by peripheral modulation of glutamate release in the locus coeruleus, the long-lasting phase by central modulation. KYNA and KMO inhibitor PNU 156561A antagonized the nicotine induced NE neuron stimulation. This extends the neuromodulatory role of KYNA via the glutamatergic to the norepinephrinic system.

Effects of KYNA on dopaminergic activity were studied with electrophysiological methods. Elevation of KYNA levels results in an increase in firing rate and bursting activity of DA neurons in both the substantia nigra (SN) and the ventral tegmental area (VTA). The partial glycine site NMDA agonist D-cycloserine was able to reverse the effects of elevated KYNA levels. NMDA

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**Dopaminergic neurotransmission**

Activity of dopaminergic neurons localized in the midbrain is proposed to be regulated by an “accelerator/brake” mechanism. The accelerator works by straightforward glutamatergic excitation of dopamine neurons. The brake is mediated through GABA-ergic interneurons. After glutamate activation in the cortex, GABA interneurons are activated. These interneurons inhibit DA activity. [1-3]

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receptor antagonist MK 801 mimicked KYNA effects. These data suggest, that the DA stimulatory effects of KYNA are mediated by acting on NMDA receptors localized on GABA interneurons ('brake mechanism'). A subchronic study confirmed the results, and showed that no adaptive mechanisms seem to exist. KYNA is also able to act in the accelerating part of DA regulation. Glutamate mediated increased nigral DA activity was effectively inhibited by KYNA. The inhibition was again mimicked by MK 801, suggesting involvement of NMDA receptors.

The delicate balance between inhibition and excitation of DA neurons was shown in a study of the effects of nicotine on DA neurons. Administration of nicotine showed an initial short lasting (<120 seconds) decrease in DA firing rate and a subsequent increase of burst firing. The decrease could be blocked by a GABA<sub>B</sub> antagonist. This suggests, that the initial decrease in DA firing rate is regulated by the brake mechanism. KYNA could effectively block the subsequent nicotine mediated increase in DA activity and even reversed it into a decreased activity. The suggested mechanism behind this observation is blockade of somatodendritic NMDA receptors on VTA DA neurons ('accelerator mechanism').

Above studies suggest a preferential action of KYNA on DA activity mediated by NMDA receptors. Involvement of a<sup>7</sup>* nAChRs has also been proven. In a set of microdialysis studies, elevated KYNA levels could reversibly reduce extracellular striatal DA concentrations. Effects were mimicked by methyllycaconitine (MLA), a selective a<sup>7</sup>* nAChR antagonist and reversed by a<sup>7</sup>* nAChR agonists choline and galantamine. NMDA receptor agonist D-serine could not reverse the effects of KYNA. The KYNA induced decrease of DA levels was therefore attributed to action through a<sup>7</sup>* nAChRs on corticostriatal glutamate neurons ('accelerator'). In a follow-up on these studies, striatal KYNA concentrations were decreased by KAT II inhibitor S-ESBA. This resulted in increased dopamine levels. Increases could be attenuated by local administration of KYNA, proving the direct role of KYNA in these effects.

### 1.1.9 Xanthurenic acid

Xanthurenic acid (XA) is the second kynurenine metabolite that is biosynthesized by kynurenine aminotransferase. The compound is synthesized from 3-OH-KYN, preferably by KAT II. The importance of XA in mammalian species is not fully understood. A recent study in rats showed, that XA crosses the blood brain barrier. It can be taken up by synaptic vesicles and neurons and the release is
depolari... suggesting a neuroregulatory role.\(^\text{169}\) XA was previously seen as a detoxicant by metabolizing 3-OH-KYN, thus preventing generation of reactive oxygen species (ROS). However, XA also has Fe-chelating properties, resulting in production of ROS.\(^\text{170}\) XA is able to complex with several substances and locally or systemically alters physiological environments. If XA levels are elevated, the compound complexes with insulin. This leads to insulin tolerance and altered glucose metabolism.\(^\text{171}\) In the kidney, XA-8-O-beta-D-glucoside and XA-8-O-sulfate function as natriuretic hormones by inhibition of sodium resorption.\(^\text{172}\) In the human lens, KAT activity and formation of XA increase with age. Oxidation of XA and binding to crystallins in the lens causes cataract.\(^\text{173}\) XA causes apoptosis by various mechanisms.\(^\text{174}\) KYN metabolism can directly be influenced by XA. At a concentration of 10\(^{-4}\)M, the compound reduced NADPH oxidation by rat KMO to 39%.\(^\text{175}\) No studies of selective inhibition of XA formation are known. Presumably, inhibition of KMO, KYNase and KAT should have effects on XA availability.

In insects, no kynureninase exists. In these species, conversion of 3-OH-KYN into XA is the major route of 3-OH-KYN clearance. The transamination occurs by an enzyme that shows only minor resemblance to KAT. The enzyme was identified as being an alanine glyoxalate aminotransferase. In insects, XA plays a role in eye pigmentation and sexual determination. The latter makes it a potential new target of malaria treatment.\(^\text{176}\)

### 1.1.10 3-Hydroxyanthranilic acid and quinolinic acid

Kynurenine metabolism by KMO and KYNase yields 3-hydroxyanthranilic acid (3-OH-AA). Like 3-OH-KYN, 3-OH-AA is able to reduce Cu(II) and Fe(III), generating superoxide and hydrogen peroxide. Copper dependent generation of ROS is involved in age related cataractogenesis.\(^\text{177}\) 3-OH-AA inhibits induction and activity of NOS II and activation of NF-cB, which is needed for NOSII gene activation.\(^\text{178}\) 3-OH-AA is the substrate for 3-hydroxyanthranilic acid oxygenase (3-HAO). 3-HAO occurs both in the periphery and in the brain. It is a monomeric cytosolic Fe\(^{2+}\)-dependent enzyme and localized in glial cells mainly.\(^\text{103}\) The enzyme can be inhibited effectively by 4-halo-3-hydroxyanthranilic acids.\(^\text{179}\) The product of 3-HAO is the unstable intermediate amino-\(\beta\)-carboxymuconic acid \(\omega\)-semialdehyde. Quinolinic acid (QUIN) is subsequently formed from the intermediate by spontaneous ring closure (Figure 5).
QUIN is an NMDA receptor agonist. It induces excitation of cortical neurons. The excitatory effects of QUIN can result in neurotoxicity, at least partly mediated by liberation of free radicals. Neuronal cell loss caused by excessive QUIN seems to be caused by damage on dendrites, rather than axons.\textsuperscript{180-182} Immune-responses can upregulate QUIN formation in microglia.\textsuperscript{183} QUIN is metabolized by quinolinic acid phosphoribosyl transferase (QRPT). Condensation with phosphoribosyl phosphate and decarboxylation affords nicotinic acid mononucleotide, finally yielding nicotinamide adenine dinucleotide (NAD, Figure 5). In the brain, like 3-HAO, QRPT is localized primarily in glial cells, but in distinct regions. QRPT is inhibited by various QUIN analogs.\textsuperscript{103}

1.2 Balances within the kynurenine pathway

A balance exists between the kynurenine pathway in the periphery and in the central nervous system. KYN is transported over the blood brain barrier via the large neutral amino acid transporter. 3-OH-KYN is transported by the same carrier, although less efficiently. QUIN, KYNA, 3-OH-AA and AA cross the blood brain barrier by passive means only. Influx rates of QUIN, KYNA and 3-OH-AA are low. The passive influx of AA only is biologically significant.\textsuperscript{33} About 60\% of brain KYN originates from the periphery. By means of intracerebral [5-\textsuperscript{3}H-KYN] injection, a functional kynurenine pathway in rat brain downstream from this intermediate was demonstrated. Because the apparent efficiency of KAT enzymes exceeds the efficiency of metabolism into the QUIN branch of the
pathway, increases in KYNA production were most pronounced. In QUIN lesioned rat striatum, activities of all enzymes of the kynurenine pathway downstream from KYN were enhanced.\textsuperscript{184} Interestingly, lesions in the early postnatal days (7 days) caused upregulation of the KYNA branch of the pathway, whereas the QUIN branch was enhanced more effectively at later ages (28 days – adulthood).\textsuperscript{185} Within the brain, kynurenine metabolism is compartmentalized. KYN is transported into astrocytes by a sodium independent large neutral amino acid transporter and into neurons by a less efficient sodium dependent transporter.\textsuperscript{112, 113} KYNA is primarily formed in astrocytes, and is removed from the brain by a probenecid sensitive carrier.\textsuperscript{136, 139} 3-OH-KYN is primarily synthesized in microglial and macroglial cells, and is further metabolized to QUIN and NAD\textsuperscript{+}.\textsuperscript{186} The functional segregation of the pathways was shown in a study using selective inhibitors. Intrastriatal [5-\textsuperscript{3}H-KYN] injection resulted in decreased formation of KYNA after selective inhibition of KAT II enzymes, without affecting production of 3-OH-KYN or QUIN. Inhibition of KMO resulted in decreased 3-OH-KYN synthesis. KYNA synthesis was not changed.\textsuperscript{53}

1.3 \textbf{Indolepyruvic acid pathway}

At least one alternative pathway for the biosynthesis of KYNA is known. Tryptophan can be transaminated by tryptophan aminotransferase or aromatic aminotransferases to indole-3-pyruvic acid (IPA). IPA is kept \textit{in vivo} in its enol-tautomeric form by means of tautomeras, probably macrophage migration inhibitory factor (MIF).\textsuperscript{187} Enol-3-IPA reacts with reactive oxygen species to form KYNA (Figure 6).\textsuperscript{188, 189}

\textbf{IPA pathway}
IPA is an inhibitor of tryptophan dioxygenase and serotonin-N-acetyltransferase.\textsuperscript{190} As summarized in ref\textsuperscript{191}, administration of IPA improved sleep in volunteers in five independent studies and had significant anxiolytic effects with concomitant improvement of sleep in a phase II trial. These effects might partly be related to production of KYNA and reduced excitation.

1.4 Kynurenine pathway: Pathology

Changes in tryptophan metabolism are involved in many pathological states. In the first place, a balance exists of tryptophan metabolism into kynurenines and towards 5-HT and related metabolites. Imbalances can cause increases or decreases in these monoamines, resulting in physiological dysfunction. Enhanced tryptophan metabolism either way can result in tryptophan depletion. Within the kynurenine pathway, imbalances between the KYNA- versus the QUIN-branch greatly influence glutamatergic neurotransmission by the inverse action on NMDA receptors of these metabolites. Furthermore, many of the kynurenines have oxidative properties, resulting in neurotoxicity. Finally, activation or inhibition of NAD production by up- or downregulation of QUIN synthesis can result in general changes in cell metabolism.

Many reviews have been published about the relationship of the KYN-pathway and physiological disorders [e.g. ref\textsuperscript{1, 10, 50, 192-196}] A short overview will be given here of the most studied pathological conditions with a main focus on CNS disorders.

1.4.1 Immune related disorders

As was stated earlier, immune responses have major effects on kynurenine pathway metabolism. In this respect, the kynurenine pathway stands in close relationship to the NO pathway.\textsuperscript{1} For example, bacterial lipopolysaccharides and IFN-\gamma induce both the kynurenine (by IDO induction) and the NO pathways in macrophages. NO inhibits IDO activity, and some of the kynurenines are able to inhibit NOS activation. Inhibition of NOS activity results in elevated IDO activity. Anti-inflammatory cytokines (IL-4 and TGF-\beta) have an inhibitory effect on IDO. Induced kynurenine metabolism leads to elevated levels of QUIN, causing immune related excitatory neurotoxicity. On the other hand, upregulation of the pathway also leads to enhanced NAD production. NAD is needed to repair immune related cell and DNA damage.\textsuperscript{1, 178, 192, 194, 197} Induced KYN metabolism causes suppression of T-cell proliferation by tryptophan depletion and direct
inhibition by 3-OH-KYN, 3-OH-AA and QUIN.\textsuperscript{198, 199} CNS infections with upregulated KYN-metabolism are polio, Lyme disease and malaria.\textsuperscript{1, 200}

\subsection*{1.4.2 AIDS-dementia complex}

Activation of the immune system by HIV infection causes activation of IDO and consequently upregulation of the kynurenine pathway. QUIN levels are elevated in patients with HIV.\textsuperscript{201} 20\% of HIV patients develop AIDS-related dementia. The cognitive and motor dysfunctions that characterize this disorder are directly related to QUIN induced neuronal damage through NMDA receptor activation. Zidovudine treatment results in lower levels of QUIN and diminished symptomatology, probably by the reduction of immune related activation of the kynurenine pathway.\textsuperscript{1, 192, 200, 202}

\subsection*{1.4.3 Huntington’s disease}

One of the (nonexcluding) hypotheses about the origin of Huntington’s disease (HD) is enhanced NMDA receptor activation, resulting in excitotoxicity (reviewed in ref\textsuperscript{203}). In animal models of HD, QUIN is able to specifically mimic the early stage symptoms. In rats, administration of QUIN induced the huntingtin gene\textsuperscript{204}, and produced the neurotoxic effects seen in HD by means of activation of NMDA receptors, oxidative stress, influencing the NO pathway and the immune system\textsuperscript{203}. In mutant \textit{mkat 2}\textsuperscript{-/-} mice, decreased KYNA levels result in higher vulnerability to QUIN.\textsuperscript{52} In human brain tissue originating from HD patients, QUIN levels were found to be elevated in the early stage of the disease, but not in later stages.\textsuperscript{205, 206} KYNA levels were decreased\textsuperscript{207, 208} or increased\textsuperscript{209} in HD patients. KAT activities were decreased.\textsuperscript{208} Moreover, 3-OH-KYN levels were elevated\textsuperscript{210} and the activity of 3-HAO was increased\textsuperscript{211}. Overall, imbalances in the kynurenine pathway might result in neurotoxicity, leading to symptoms seen in patients with HD.

\subsection*{1.4.4 Alzheimer’s disease and cognitive function}

Norepinephrine release in the locus coeruleus as a response to NMDA receptor activation is an important regulator in cognitive function. Elevated levels of KYNA are believed to impair cognitive function by NMDA antagonism. In fact, some of the known cognitive enhancers directly counteract the antagonistic action of KYNA on the NMDA receptor.\textsuperscript{5}

In Alzheimer’s disease, an elevated KYN/Trp ratio was found, implicating enhanced Trp metabolism.\textsuperscript{212} KYNA levels were increased in the brain of AD
patients, correlating with increased KAT activity.\textsuperscript{213} In serum and red blood cells, KYNA levels were decreased.\textsuperscript{214} 3-OH-KYN levels were unchanged.\textsuperscript{210} Immune responses, e.g. to β-amyloid plaques, are thought to upregulate Trp metabolism via the kynurenine pathway. Whereas increased KYNA levels might lead to cognitive impairment seen in AD, neurotoxic kynurenines might cause further brain damage.\textsuperscript{196}

\subsection*{1.4.5 Parkinson’s disease, epilepsy}

Mitochondrial complex I inhibitor MPTP causes decreased KYNA availability by KAT depletion in the substantia nigra, directly linking a widely used model of Parkinson’s disease to the kynurenine pathway.\textsuperscript{123, 124} In patients suffering from Parkinson’s disease (PD), elevated levels of 3-OH-KYN and a decreased KYN/3-OH-KYN ratio in the putamen and substantia nigra have been found. KYNA levels were decreased in these areas in PD patients with and without L-DOPA treatment.\textsuperscript{215} Interestingly, diskenysia as a side-effect of L-DOPA treatment could effectively be treated by increasing KYNA levels.\textsuperscript{216} In conclusion, shifts in KYN metabolism towards the QUIN branch might have detrimental effects on Glu and DA release and might cause direct neurotoxicity in the substantia nigra. Pharmacological manipulation of the KYN pathway should be regarded as a possible target for treating symptoms of PD or at least side effects of current treatment.

The discovery of convulsant properties of QUIN led to the first steps in accepting a physiological function of the kynurenine pathway instead of solely being a route of Trp catabolism.\textsuperscript{100} Excessive NMDA receptor stimulation resulting in overexcitation is a cause of epileptic seizures. Soon, the knowledge of the physiological effects of KYNA and QUIN on the NMDA receptor led to experimental tools to be used in the attenuation of epileptic seizures. 4-Chlorokynurenine is converted \textit{in vivo} into 7-Cl-KYNA by KATs. Like KYNA, 7-Cl-KYNA is an antagonist at the glycine site of the NMDA receptor and could attenuate epileptic symptoms evoked by the chemoconvulsant kainate in rats.\textsuperscript{217, 218} KMO inhibitors have been published which were shown to have sedative and anticonvulsive properties by producing elevated KYNA levels in brain, blood, liver and hippocampal microdialysate in rats.\textsuperscript{41} Clearly, shifting the balance of the KYN pathway towards neuroprotective KYNA might be an alternative strategy for achieving NMDA antagonism in the treatment of epilepsy.
1.4.6 Schizophrenia

In recent years, the dopamine hypothesis of schizophrenia has been extended to a broader hypothesis of dysregulation of neurotransmitters in the brain. An important aspect in this view is the hypothesis of glutamatergic hypofunction. In short, this hypothesis proposes a glutamate deficiency. Dopamine release in the mesolimbic region is controlled by inhibitory GABAergic interneurons, which are stimulated by Glu neurons. Hypoglutamatergic tone results in elevated DA levels in these regions. Elevated mesolimbic DA levels are mainly associated with positive symptoms of schizophrenia. The mesocortical DA transmission is directly stimulated by glutamate. Hypoglutamatergic tone therefore results in reduced DA levels in the PFC. Reduced levels of DA in the PFC are mainly associated with negative symptoms and cognitive impairment.219-222 The use of non-competitive NMDA antagonists to evoke schizophrenic symptoms is a commonly accepted model for the disorder. (Sub)chronic PCP treatment most closely mimics positive, negative and cognitive symptoms and concurrent physiological changes.223 In close relationship to the hypoglutamatergic hypothesis stands the KYNA hypothesis of schizophrenia, stating that elevated levels of this compound might play a role in the pathophysiology of the disorder. Elevated levels of KYNA have been found in cerebrospinal fluid (CSF) and brain of patients suffering from schizophrenia.2-4 In a study comparing blood levels of patients (85% of which received antipsychotic treatment) and healthy volunteers, levels of tryptophan, KYN and KYNA did not show any differences. The KYN/Trp ratio was significantly higher in patients, indicating enhanced tryptophan metabolism into the kynurenine pathway.224 KYNA is able to reproduce many of the symptoms seen in schizophrenia.225 Acute and subchronic exposure to KYNA disrupts prepulse inhibition in rats, a model of sensorimotor gating.163, 226 Elevated levels of KYNA increased neuronal firing of dopamine neurons in the VTA in rats (see section 1.1.8.3).162 The atypical antipsychotic clozapine was able to decrease this KYNA induced burst firing of VTA DA neurons.163, 227 Although effects could not completely be explained by action on NMDA receptors, KYNA was shown to disrupt auditory processing in rats.228 Spatial working memory deficits seen in schizophrenic patients could also be reproduced by elevated KYNA levels.229 Interestingly, chronic treatment with either typical (haloperidol, raclopride) or atypical (clozapine) antipsychotics reduced rat brain KYNA levels.152 The cause of elevation of KYNA levels in schizophrenia is not completely understood. Evidence suggests upregulated tryptophan metabolism with an
inflammatory background. The immune response was identified as a type 2-immune activation. In this type of response, astrocytic TDO rather than IDO is upregulated. In accordance with upregulation of the initial step of Trp metabolism, the KYN/Trp ratio was significantly higher in schizophrenic patients than in controls.\textsuperscript{224, 230} COX-2 inhibitors are able to shift the balance of type 1/type 2 immune responses towards the type 1 response by inhibition of prostaglandin E\textsubscript{2} release. In this respect, it is interesting to know that COX-2 inhibitors were also shown to decrease KYNA levels in rats.\textsuperscript{150, 230} Downstream of kynurenine, elevated KAT I activities were observed in post mortem brain samples of schizophrenic patients.\textsuperscript{231}

### 1.4.7 Other disorders

Other disorders in which dysregulation of KYN pathway metabolites has been observed include depression, traumatic CNS injury/ischemic damage, tumor growth, hepatic encephalopathy, diabetes, gastrointestinal tract disorders, (age related) cataractogenesis, hypertension, and renal failure.\textsuperscript{1, 10, 90, 177, 193, 195, 200, 202, 230, 232}

### 1.5 Conclusion

In conclusion, the kynurenine pathway is a metabolic pathway that produces physiologically active compounds and plays a role in regulation of brain function. The pathway is highly balanced around key intermediate KYN. Depending on the specific environment, the pathway can be balanced towards glutamatergic agonism (QUIN) or antagonism (KYNA). In general, the enzymes that are involved in the pathway are known. However, much work needs to be done on detailed characterization of most of the enzymes. An important issue in this context is the difference that seems to exist between different species.

Concerning drug design, the major point of interest is finding pharmacological tools that can shift the KP balance towards one of the two branches - the KYNA or the QUIN branch. In most cases, a shift towards the neuroprotective KYNA branch is desired. Potent inhibitors of KMO and KYNase are known. Indeed, these compounds are able to shift the pathway towards the KYNA branch. Compounds that are known to inhibit the KYNA branch and are able to upregulate the QUIN branch are less abundant. Characterization of the physiological effects of these compounds is ongoing. Again, species differences, as well as brain region specific effects are issues of consideration.
Being embedded in the complex network of brain functions, dysregulation of the kynurenine pathway can be related to many CNS disorders. Whether imbalances in this pathway are the cause or the consequence or even both of the disorder, is mostly unknown. Ultimately, research in the field of pharmacological manipulation of the kynurenine pathway balances should lead to new methods for treatment of (CNS) disorders.

1.6 Aim of thesis

The aim of this work was to find pharmacologically active compounds that shift the kynurenine pathway away from KYNA synthesis. With such compounds, effects of decreasing KYNA levels could be studied under physiological and pathological circumstances. Whereas inhibitors of the QUIN branch are widely available, only few compounds are known that inhibit the KYNA branch of the pathway.

For this purpose, different classes of compounds were synthesized. The in vitro inhibitory activity on rat brain KATs was examined. Furthermore, selectivity towards KAT over KYNase and KMO was taken into account. Since the latter two enzymes are most abundant and active in the liver, rat liver homogenate was used for examination of inhibitory activities of compounds.

In chapter 2, the synthesis and in vitro properties of conformationally restricted carboxylic acids are described. In these compounds, the KYN core structure is incorporated in a bicyclic ring system. For synthetic feasibility and examination of the role of the KYN α-amino group, this group is not included in the analogs. In chapters 3 and 4, the corresponding bicyclic amino acid analogs are presented. The synthesis of these compounds is described in detail. In vitro activity is discussed and related to structural variation. In chapter 5, four fully flexible KYN analogs are presented. These compounds were synthesized to examine the effect of omittance of the α-amino group on KAT inhibition and selectivity with and without substitution of the aromatic ring. Furthermore, the effect of bioisosteric replacement of the carboxylate group was examined. The in vitro profiles of these compounds elucidate some interesting features of activity and selectivity.

Chapter 6 reviews the current knowledge of kynurenine aminotransferases. KAT II homology models are presented and discussed in the light of recently published crystal structures. In the future, the structural knowledge of KP enzymes should provide easily accessible tools for design of new compounds.
In chapter 7, the current status of research in the kynurenine pathway is discussed, taking into account the work presented in chapters 2-6. Concluding remarks are given on current and future work in the area.

1.7 References


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