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Advantages of brain penetrating inhibitors of kynurenine-3-monooxygenase for treatment of neurodegenerative diseases

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Abstract

Kynurenine-3-monooxygenase (KMO) is an important therapeutic target for several brain disorders that has been extensively studied in recent years. Potent inhibitors towards KMO have been developed and tested within different disease models, showing great therapeutic potential, especially in models of neurodegenerative disease. The inhibition of KMO reduces the production of downstream toxic kynurenine pathway metabolites and shifts the flux to the formation of the neuroprotectant kynurenic acid. However, the efficacy of KMO inhibitors in neurodegenerative disease has been limited by their poor brain permeability. Combined with virtual screening and prodrug strategies, a novel brain penetrating KMO inhibitor has been developed which dramatically decreases neurotoxic metabolites. This review highlights the importance of KMO as a drug target in neurological disease and the benefits of brain permeable inhibitors in modulating kynurenine pathway metabolites in the central nervous system.

Kynurenine-3-monooxygenase

Kynurenine 3-monooxygenase (KMO, EC 1.14.13.9) is an NADPH-dependent flavin monooxygenase which catalyses the hydroxylation of the L-tryptophan (TRP) metabolite L-kynurenine (L-KYN) into 3-hydroxykynurenine (3-HK). KMO is expressed in microglia and infiltrating macrophages in the brain [1], and at high levels in the liver, kidneys and placenta in the periphery [2]. Specifically, KMO is localised to the outer membrane of mitochondria [3] where it associates with the lipid membrane using C-terminal transmembrane domains, which are also crucial for the catalytic activity of KMO [4]. The cofactor flavin adenine dinucleotide (FAD) binds to KMO at a 1:1 ratio [5]. Following binding of L-KYN to KMO (Figure 1), NADPH acts as an electron donor and reduces FAD, leading to the formation of a L-KYN-FAD-hydroperoxide intermediate. L-KYN is then oxidised, resulting in the release of 3-HK and water. KMO is an important enzyme in the kynurenine pathway (KP, Figure 2),

Competing Interests statement

A patent (new compounds and uses) from the University of Leicester and University of Manchester by N.S.S. and F.G is pending, application number 1719327.7. The authors declare there are no other competing financial interests.

the major catabolic route of TRP, and lies at a critical branchpoint of the KP, which makes it an attractive drug target for several neurodegenerative and neuroinflammatory diseases [6].

Overview of the kynurenine pathway

The KP (Figure 2) metabolises more than 95% of TRP [7]. This pathway has been implicated in numerous diseases, including Huntington's disease, Alzheimer's disease, Parkinson's disease, schizophrenia, acute pancreatitis and cancer [8–11]. Hence, there is an increasing interest in identifying new therapeutic strategies by targeting the KP. The KP metabolises TRP into a number of neuroactive metabolites, such as kynurenic acid (KYNA), 3-HK, quinolinic acid (QUIN), xanthurenic and cinnabarinic acids (Figure 2). The role of the KP metabolites in several neurological disorders has been investigated intensively in recent years. Each of the metabolites appears to exert different effects: KYNA is a neuroprotectant [12, 13], 3-HK generates free radicals [14], QUIN is primarily an excitotoxin [15], and xanthurenic and cinnabarinic acids activate metabotropic glutamate receptors [16].

In the KP, the initial step is the conversion of TRP to N-formyl-L-kynurenine through indoleamine 2,3-dioxygenase 1 and 2 (IDO1 and IDO2) or tryptophan 2,3-dioxygenase (TDO) [17]. This results in the synthesis of L-KYN, which can be metabolised by three different enzymes and lies at the key branchpoint of the KP [18]. L-KYN can be metabolised to 3-HK by KMO, or it can form KYNA by a transamination reaction catalysed by kynurenine aminotransferase II (KATII), or alternatively it can be converted to anthranilic acid (AA) by kynureninase, which then feeds back into the 3-HK branch of the KP. Since KMO has the tightest binding affinity for L-KYN under normal conditions, the KMO branch has been considered to be the major metabolic route of the KP [18, 19]. KMO activity plays an essential role in maintaining a balance between the neurotoxic and neuroprotective potential of the pathway. Hence, there has been a focus on KMO inhibition as a potential strategy to treat several neurodegenerative and neuroinflammatory diseases.

KMO inhibition and neurodegenerative diseases

Huntington's disease

Huntington's disease is a fatal neurodegenerative disorder which is inherited in an autosomal dominant manner [20]. In the early stages of disease patients often exhibit changes in mood or cognitive ability. As the disorder develops the most characteristic physical symptoms (chorea) will appear. Huntington's disease is caused by a CAG trinucleotide repeat expansion in the *HTT* gene, leading to the expansion of a polyglutamine tract in the huntingtin protein (HTT). These mutant forms of HTT can misfold and aggregate, eventually resulting in neuronal cell death [21]. Evidence suggests that the KP may contribute to the neurodegenerative effects observed in patients. Increased levels of the potentially neurotoxic metabolites 3-HK and QUIN have been detected in tissues taken from early stage patients [22–24] and several different studies indicate that there are significantly increased levels of these metabolites in mouse models of Huntington's disease [25, 26], indicating dysregulation of the KP in Huntington's disease. Notably, studies using yeast and *Drosophila* models of Huntington's disease discovered that genetic inhibition of KMO

normalised KP metabolic imbalances and ameliorated disease-related phenotypes [27–29]. Furthermore, pharmacological inhibition of KMO in a *Drosophila* model of Huntington's using several KMO inhibitors protected against neuron loss [27, 28, 30]. The KMO inhibitor JM6 has also been shown to reduce loss of synapses and prevent microglial activation in a mouse model of Huntington's disease [31], while the KMO inhibitor CHDI-340246 was found to ameliorate electrophysiological disturbances [32].

Alzheimer's disease

Alzheimer's disease is the most common cause of dementia [33]. It is a chronic neurodegenerative disease that usually develops slowly and causes gradual deterioration over time. The earliest symptom is regularly forgetting recent events and becoming increasingly repetitive. At later stages, patients develop problems with language, thinking and disorientation, as well as a number of behavioural issues. Post-mortem Alzheimer's brains exhibit extracellular formation and accumulation of senile plaques, primarily comprised of misfolded amyloid-β peptides and also intracellular phosphorylation of tau proteins leading to the formation of neurofibrillary tangles [34]. Alzheimer's patients tend to exhibit decreased TRP levels, which is possibly due to the increased accumulation of KP metabolites [35]. QUIN has been shown to co-localise with neurofibrillary tangles in cerebral neurons of Alzheimer's patients, modulate tau phosphorylation [36] and promote tau aggregation[37]. Abnormal IDO1, 3-HK, and QUIN levels have been correlated with specific stages of Alzheimer's disease [38–40]. It has been demonstrated that treatment with a KMO inhibitor in a mouse model of Alzheimer's disease significantly prevented synaptic loss and improved spatial memory [31]. Down regulation of KMO gene expression ameliorates several disease-relevant phenotypes in Alzheimer's model flies, including neurodegeneration, locomotor abnormalities and shortened lifespan [29].

Parkinson's disease

Parkinson's disease is a long-term progressive neurological disorder [41] which mainly affects the motor system in the central nervous system (CNS). Early in the disease, the common symptoms are shaking, rigidity and slowness of movements, with the potential onset of dementia in advanced stages. The cause of Parkinson's disease is believed to involve both genetic and environmental factors. In addition to more than 20 genes being associated with familial forms of Parkinson's, meta-analyses of several genome-wide association studies (GWAS) have revealed at least an additional 90 independent genetic risk factors [42, 43]. Notably, the gene encoding the KP enzyme aminocarboxymuconate-semialdehyde-decarboxylase (*ACMSD*) has been implicated in Parkinson's via several genetic studies[44]. Similarly to Huntington's and Alzheimer's diseases, Parkinson's disease is a protein misfolding disorder. Indeed, misfolding and aggregation of α-synuclein, which is the major component of Lewy bodies, is associated with Parkinson's pathogenesis [45, 46]. In a manner similar to tau, QUIN has been found to seed aggregation of α-synuclein[47], which may have ramifications on Parkinson's pathogenesis. Parkinson's disease is also associated with oxidative stress, mitochondrial dysfunction, excitotoxicity and inflammation, which again show correlations alterations in KP metabolites [45, 48]. In the basal ganglia of Parkinson's disease patients, an increased concentration of 3-HK has been found, while L-KYN and KYNA levels are slightly reduced [49, 50]. Increased levels

of 3-HK and L-KYN have also been detected in the cerebral spinal fluid of patients with Parkinson's disease [51]. The inhibition of KMO dramatically decreases the 3-HK/KYNA ratio in *Drosophila*, ameliorating disease phenotypes in Parkinson's model flies [29]. Thus, KMO inhibition is a promising therapy for Parkinson's disease.

Biochemistry and biophysical studies aid the development of brain penetrating inhibitors of KMO

Due to the lack of any structural information at the time, early KMO inhibitors were mainly based on analogues of L-KYN. An array of substrate analogue inhibitors showing therapeutic potential, has been identified, such as m-nitrobenzoylalanine (m-NBA), 3,4-dichlorobenzoylalanine and UPF-648 [52–55]. However, kinetics studies of *Pf*KMO reveal that these substrate analogue inhibitors actually act as effector molecules. Binding of these compounds to KMO stimulates the flavin reduction by NADPH and causes the generation of cytotoxic hydrogen peroxide [56].

More specific and potent KMO inhibitors have been sought from novel and more complex organic compounds. Based on the L-KYN carboxyl group bioisosteres chemical library, a series of N-(4-phenylthiazol-2-yl) benzenesulfonamides KMO inhibitors has been developed [57]. Among these, oral administration of Ro 61–8048, increased the KYNA concentration in the brain by 7.5 fold at a dose of 100 μM/kg [57]. However, Ro 61–8048 shows poor brain permeability in mice, indicating that the protective effects generated by Ro 61–8048 are possibly due to the inhibition of peripheral KMO. The increased L-KYN levels in blood can then be transported across the blood-brain barrier (BBB) into the brain and converted into neuroprotective KYNA. A prodrug (which will be metabolised to the active drug *in vivo*) derived from Ro 61–8048, named JM6, has also been produced and found to be efficacious [31], although a later study has shown that JM6 is not a prodrug of Ro 61–8048 [58].

Elucidation of the KMO crystal structure and detailed kinetic studies have accelerated the development of KMO inhibitors [8, 56, 59]. A series of novel compounds has been identified that lead to inhibition of KMO activity. KMO inhibitors with IC_{50} in the nM region have been identified [8, 30, 60, 61]. Nonetheless, poor brain permeability has limited the application of KMO inhibitors within the CNS. KMO inhibitors with brain permeability would be predicted to be more efficacious for treating neurodegenerative diseases than peripheral treatment, as inhibition of KMO in the CNS leads to increased neuroprotective KYNA levels as well as decreased levels of neurotoxic metabolites [30, 62, 63]. To this end, the KMO structure and ligand interaction data were used as a basis for virtual screening and combined with a prodrug strategy to develop brain-permeable KMO inhibitors [30]. Prodrugs are considered as one of the most promising technologies for lead compound optimisation to cross the BBB. For KMO inhibitors, one of the main challenges to cross the BBB is the acidic centre, which mimics the binding of the carboxyl group of L-KYN. In the KMO–inhibitor 1 structure, the carboxylate group of the inhibitor sits close to residues R83/Y97/N368 in the KMO active site (Figure 3). Several interactions between ligand and protein have been identified (Figure 3). This network of polar interactions between protein and ligand makes the compound a potent KMO inhibitor. The complex structure also

indicates that the carboxylate region of the inhibitor could be modified as part of a prodrug strategy. By esterification of the carboxyl group, the brain permeability of KMO inhibitor **1** was improved, and a maximal brain:blood ratio of 3.22 at 15 min was achieved[30]. As endothelial cells (ECs) in the CNS are joined together with highly resistant tight junctions, which prevents the passage of polar solutes [64], it is not surprising that esterification of KMO inhibitors could improve its brain permeability. Chemically modifying a drug to become more lipophilic is also a widely used approach to improve BBB penetration of other carboxylic acid containing drugs [65].

As the brain ECs constrain the movement of molecules between the blood and brain, overcoming the BBB remains a challenge for the treatment of CNS disorders. Indeed, the efficacy of traditional oral or systemic administrations of therapeutic drugs is limited by their poor brain permeability. Several strategies have been developed to overcome the issue, with chemical modifications to generate derivatives of a drug proving to be an effective way to improve brain penetration. The novel drug should possess the optimal physicochemical properties to permit passive diffusion, or on the other hand could be recognised as a "substrate" to utilise the endogenous transport systems of the BBB [64]. The previously reported brain penetrating KMO inhibitor (Compound **1**) has both characteristics; it is relatively small and lipid-soluble, and the cheminformatics and *in vitro* cell assays indicate that it can cross the BBB via riboflavin transporters (see below), which are expressed in most of the tissues [30]. For the design of future KMO inhibitors, the polarity and similarity with existing endogenous metabolites could be used as a guide to achieve brain permeability.

The advantages of brain penetrating inhibitors of KMO

The BBB comprises microvascular ECs surrounded by pericytes and the end feet of astrocytes. The apical side of the ECs is in contact with the blood and forms the capillary lumen, whereas the basolateral side is in contact with glial cells of the brain. Several characteristics of the BBB limit the uptake of molecules into the CNS, therefore protecting the brain from toxins and pathogens. Unlike the normal vascular endothelium which allows diffusion of molecules between cells and into tissues, ECs of the BBB are held closely together by tight junctions. These prevent diffusion of most molecules between the ECs across the BBB and into the brain [66, 67]. In addition, low rates of transcytosis by ECs of the BBB reduce vesicle-mediated movement of molecules across the BBB [66]. Although these mechanisms protect the brain, these characteristics of the BBB also prevent the delivery of 98% of small-molecule drugs into the brain [68], limiting the delivery of drugs into the CNS to treat neurological conditions.

Targeting KMO inhibitors to the CNS where they can directly inhibit KMO is preferable to targeting peripheral KMO (Figure 4), as this reduces production of 3-HK and downstream metabolites in the brain and promotes increased levels of neuroprotective KYNA [30]. In general, for drugs to cross the BBB they must have a molecular mass <400 Da and have <8 hydrogen bonds to be lipid soluble [68]. However, efflux pumps expressed on ECs often remove drugs that have transferred from the blood across the plasma membrane of ECs in the BBB [69, 70]. A mechanism used to target drugs into the CNS is to design drugs that can be transported across the BBB by uptake transporters which allow essential nutrients to pass

into the brain [71]. The recently developed brain penetrating KMO inhibitor (Compound **1**) was shown to cross the BBB using specific uptake transporter proteins expressed in ECs of the BBB known as riboflavin transporters [30]. The SLC52 family of riboflavin transporters are expressed in the brain [72] and transport riboflavin across the plasma membrane [73]. A separate study also identified a riboflavin transporter expressed by microvascular brain ECs that transported riboflavin from the apical to basolateral side of the ECs [74]. The transport of the KMO inhibitor prodrug across the BBB by a riboflavin transporter was supported by the fact that the prodrug out-competed riboflavin uptake in a leukaemia cell line, and the KMO inhibitor prodrug was also shown to release functional KMO inhibitor in the brains of rats shortly after intravenous administration [30]. Crucially, peripheral administration of the prodrug in rats significantly reduced levels of 3-HK in the brain within 1 h, whereas intravenous administration of the KMO inhibitor Ro 61–8048 - which cannot cross the BBB - had no effect on 3-HK levels in the brain [30]. This study shows promising results for the development of a brain permeable KMO inhibitor that could potentially be used to target KMO in the CNS and directly reduce levels of neurotoxic 3-HK and other neurotoxic downstream metabolites of the KP. The development of a brain permeable KMO inhibitor would therefore allow treatment of neurological disorders for which there are currently no available drugs that can enter the CNS.

Future perspectives

The KP regulates many important physiological processes and plays an adverse role in several pathological states, such as neurodegenerative and neuroinflammatory diseases, cancer and chronic infection [8–11]. The inhibition of KMO has been found to normalise pathological KP imbalances in several disease models, ameliorating disease phenotypes [31, 75–78]. The development of potent KMO inhibitors has become straightforward due to the availability of the protein structure and the ease of functional studies. The next challenge is how to improve its clinical behaviour to avoid side effects and have a better efficacy. A brain permeable inhibitor would be highly desirable as it can prevent the accumulation of neurotoxic metabolites in the brain, while increasing levels of neuroprotective KYNA. In addition, several KMO inhibitors are based on L-KYN analogues, and most of these work as effectors rather than a true inhibitor, which can stimulate the reduction of flavin and produce cytotoxic hydrogen peroxide [30, 56, 79]. Thus, in future KMO inhibitor development, these effector type inhibitors should be avoided and for CNS disorders brain permeability should also be enhanced. Regarding the brain permeable KMO inhibitor (Compound **1**) [30], thorough testing in different disease models is now required to validate its therapeutic potential. Modification of the inhibitor to improve the efficacy should also be considered.

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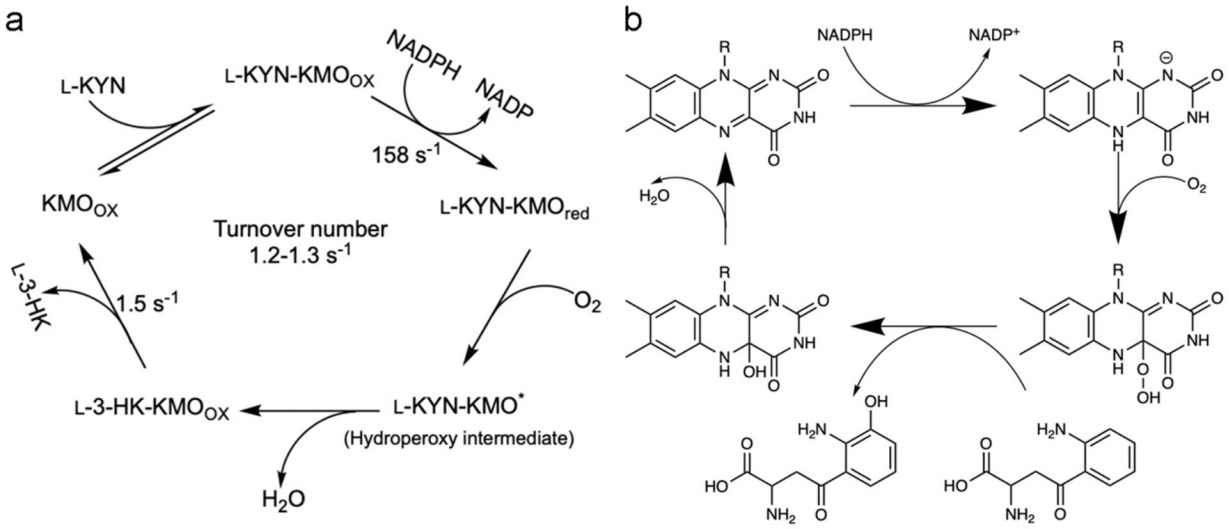

Figure 1. The KMO reaction cycle.

In the KMO reaction, L-KYN binds to the protein first, then NADPH binds and reduces the FAD followed by $NADP^+$ release, allowing for dioxygen binding. A FAD–superoxide radical pair is likely to be formed and rapidly decays to a peroxy-flavin structure, which will act as the electrophile for L-KYN oxidation. C4a-hydroxy flavin is then dehydrated yielding the oxidised flavin. The final step is 3-HK release, which is also the rate limiting step with a slightly faster rate than the overall turnover number [56].

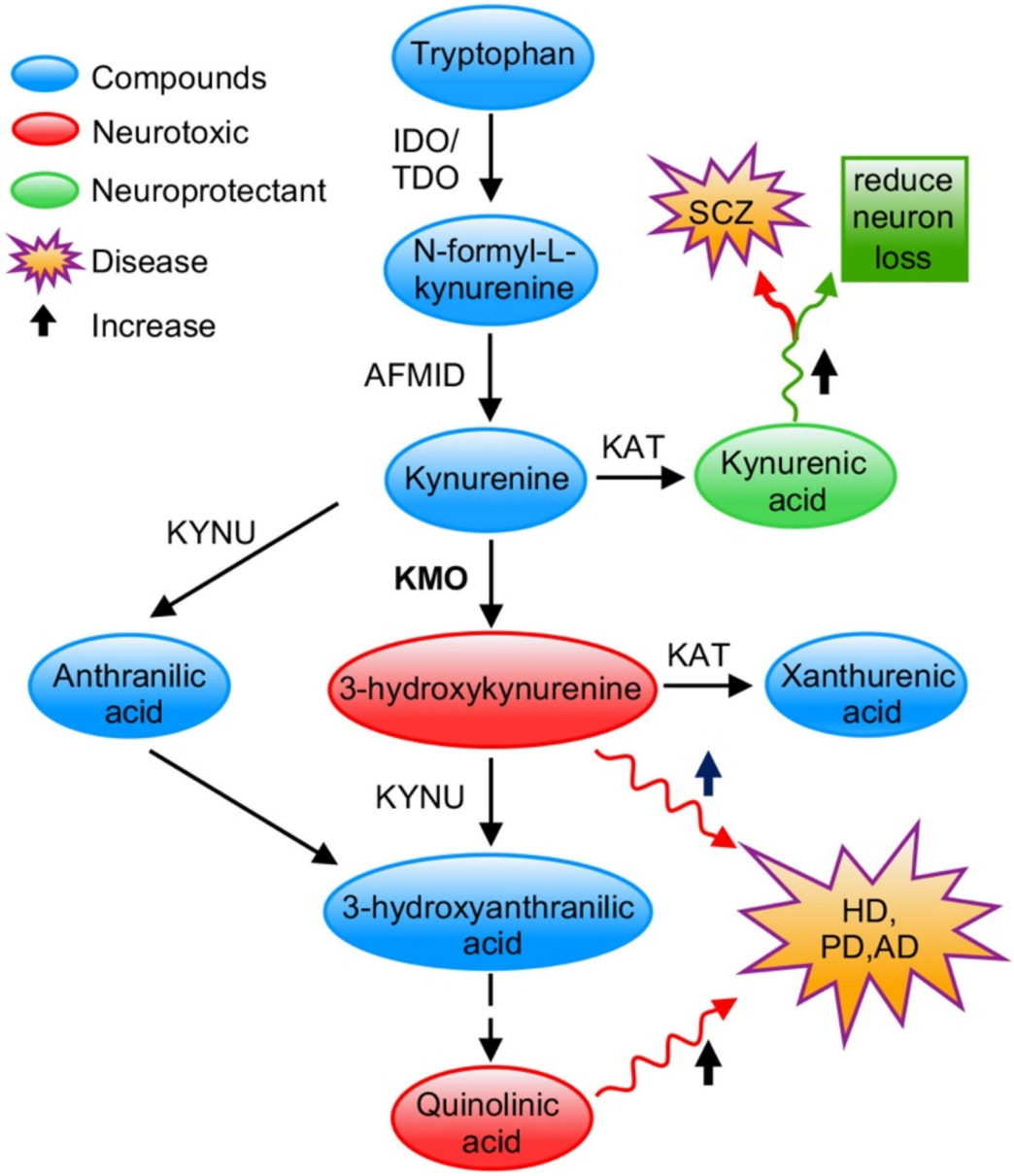

Figure 2. Overview of kynurenine pathway.

IDO, indole- 2,3-dioxygenase; TDO, tryptophan-2,3- dioxygenase; AFMID, Arylformamidase; KAT, kynurenine aminotransferase; KYNU, kynurenase; HD, Huntington's disease; PD, Parkinson's disease; AD, Alzheimer's disease; SCZ, Schizophrenia. Neurotoxic metabolites 3-hydroxykynurenine and quinolinic acid are highlighted in red. Neuroprotective kynurenic acid is highlighted in green. Their relation with neuro disorders is labelled in the figure.

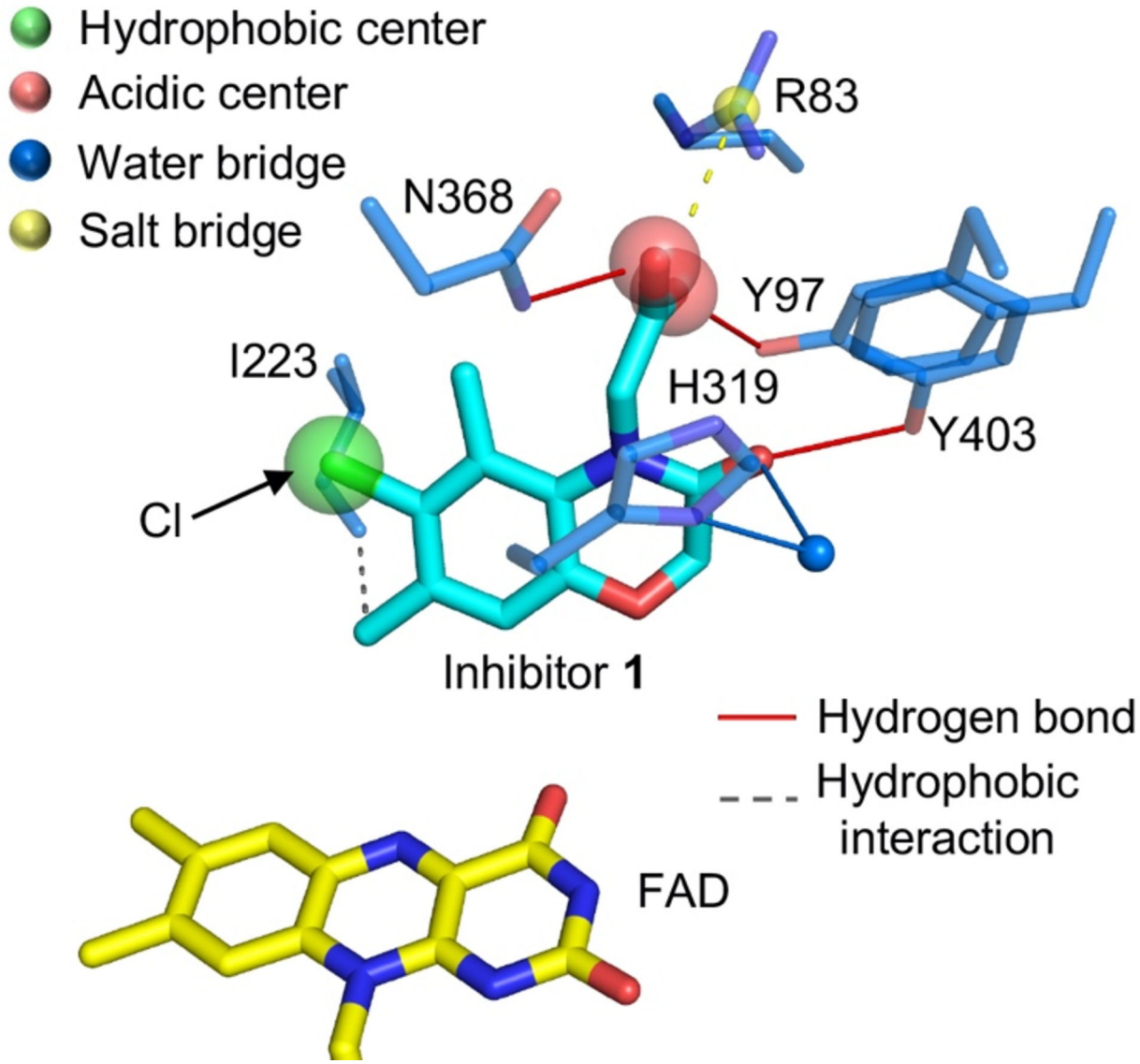

Figure 3. Structure basis of brain penetrating KMO inhibitors.

The interaction between compound **1** and *Pseudomonas fluorescens* KMO [30] (Structure file acquired from Protein Data Bank, 6FP1, https://www.rcsb.org). Key active site residues are shown in atom coloured sticks, carbons of KMO in blue, inhibitor 1 in cyan and FAD in yellow.

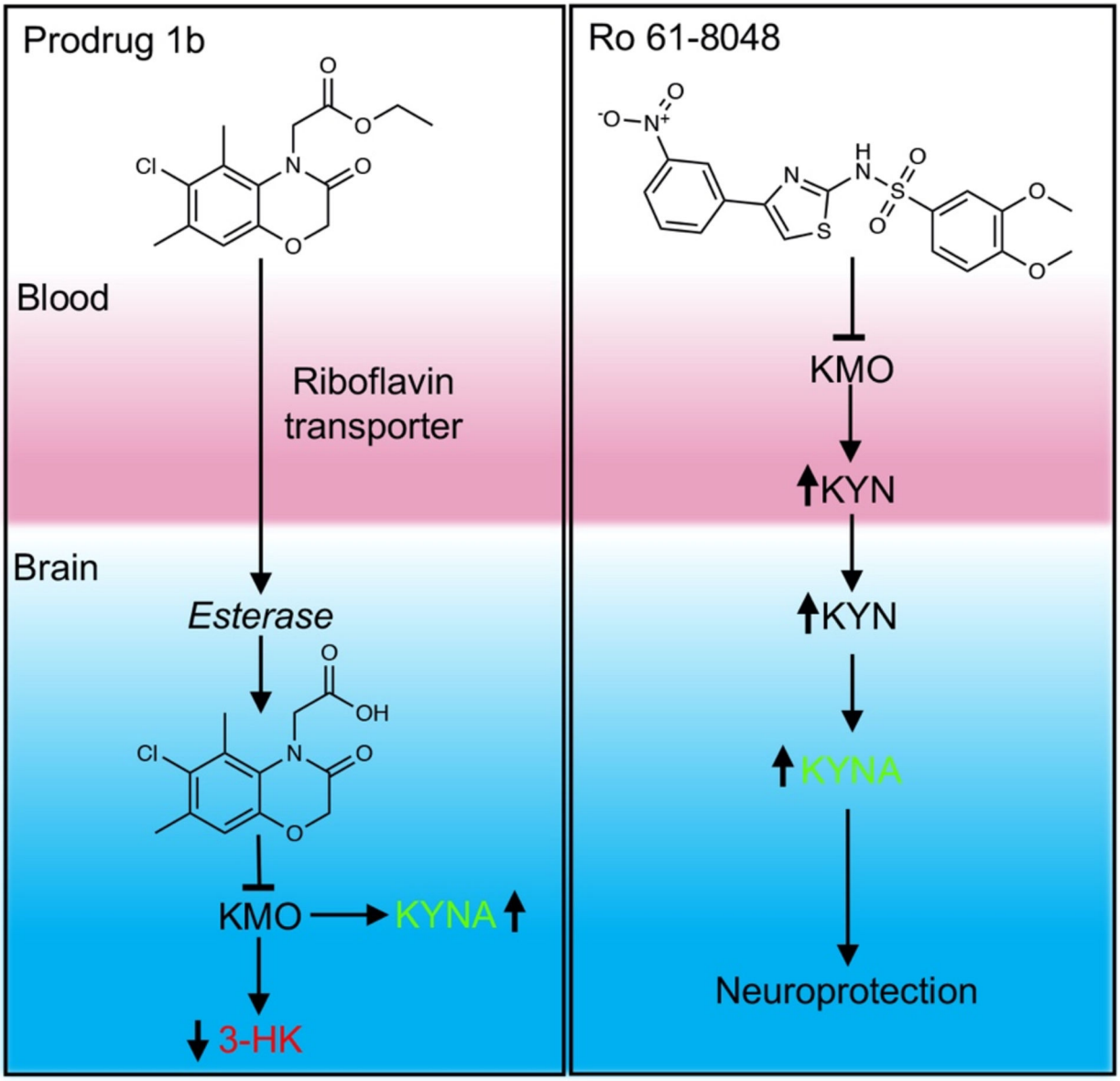

Figure 4. Advantages of brain penetrating KMO inhibitors.

Brain penetrating KMO inhibitor transferred into brain and activated by esterase. The activated KMO inhibitor suppresses the activity of KMO in brain, leading to the decrease of neurotoxic 3-HK and increase of neuroprotective KYNA. While the non-brain penetrating inhibitors act in the periphery, raising levels of L-KYN in the blood. The L-KYN will be transported to brain and converted to neuroprotective KYNA.