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Development of a microplate fluorescence assay for kynurenine aminotransferase

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ABSTRACT

Inhibition of kynurenine aminotransferases (KATs) is a strategy to therapeutically reduce levels of kynurenic acid (KYNA), an endogenous antagonist of glutamatergic *N*-methyl-D-aspartate (NMDA) and cholinergic α_7 nicotinic receptors. Several methods of measuring KAT activity in vitro have been developed, but none is well-suited to high throughput and automation. In this article, we describe a modification of existing high-performance liquid chromatography (HPLC)-based methods that enables the development of a 96-well microplate assay in both enzyme- and cell-based formats using human KAT I as an example. KYNA enzymatically produced from L-kynurenine is measured directly in a reaction mixture fluorimetrically.

Dysregulation of the kynurenine pathway of tryptophan metabolism in the brain has been linked to multiple neurodegenerative and psychiatric diseases. In these disorders, either increased or decreased levels of kynurenic acid (KYNA)[1] are correlated with various disease states. Because KYNA is an antagonist of both the α_7 nicotinic acetylcholine receptor and the *N*-methyl-D-aspartate (NMDA) glutamate receptor [1], altered KYNA levels could signal dysregulation of neuronal excitability, synaptic plasticity, or neuroprotection. Therefore, modulating this pathway using small molecule inhibitors is an emerging strategy for the treatment of a variety of conditions. Existing strategies, such as kynurenine hydroxylase (KMO) inhibition, are focused on increasing KYNA as a potential neuroprotective approach for diseases such as Huntington's disease, Parkinson's disease, ischemia, and epilepsy [2]. However, chronically increased levels of KYNA can potentially lead to hypofunction of glutamatergic and cholinergic neurotransmission, as has been proposed to occur in Alzheimer's disease [3], schizophrenia [4,5], and Down syndrome [6]. Thus, it is important to develop and understand the properties of inhibitors that reduce KYNA.

KYNA is a product of the irreversible transamination of L-kynurenine (L-KYN) catalyzed by kynurenine aminotransferases (KATs). Hence, inhibition of KATs is an attractive strategy for normalization of increased levels of KYNA in the diseased brain. Currently, there are four KATs in human and rodent brains, with KAT I and KAT II being the most studied [7]. The ability to pharmacologically modulate KATs has been limited, and only a rat-specific KAT II inhibitor with poor potency had been reported [8] until recently. A different rat KAT II inhibitor was discovered recently [9], and it was reported to also have activity against human KAT (huKAT) II [10]. The discovery of KAT inhibitors has been hampered by the inability to use existing high-performance liquid chromatography (HPLC)-based KAT assays for high-throughput screening (HTS). KAT I was initially identified as glutamine transaminase K (GTK) and later was shown to be identical to cysteine *S*-conjugate β-lyase (CCBL1) (for a review, see Ref. [11]). An absorbance-based KAT I (GTK, CCBL1) assay was developed around this enzyme's ability to transaminate L-phenylalanine (L-Phe) to a light-absorbing phenylpyruvate (322 nm, $\varepsilon = 2.4 \times 10^4$ in 3 M NaOH) [12]. The major disadvantages of this assay are the following: many compounds absorb at this wavelength, the assay cannot be used for assaying other KATs that do not transaminate L-Phe, and the assay requires highly concentrated NaOH for detection. Here, using huKAT I as an example, we present the development of a simple HTS-compatible microplate fluorescence-based KAT enzymatic assay that enables the discovery of potential drugs for neurological diseases and tools for further elucidation of the role of the kynurenine pathway in brain function.

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[1] *Abbreviations used:* KYNA, kynurenic acid; NMDA, *N*-methyl-D-aspartate; KMO, kynurenine hydroxylase; L-KYN, L-kynurenine; KAT, kynurenine aminotransferase; huKAT, human KAT; HPLC, high-performance liquid chromatography; HTS, high-throughput screening; GTK, glutamine transaminase K; CCBL1, cysteine *S*-conjugate β-lyase; L-Phe, L-phenylalanine; AMP, 2-amino-2-methyl-1-propanol; PLP, pyridoxal-5′-phosphate hydrate; Na pyruvate, sodium pyruvate; ZnOAc, zinc acetate; NaOAc, sodium acetate; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; KRB buffer, Krebs–Ringer bicarbonate buffer; cDNA, complementary DNA; DMSO, dimethyl sulfoxide; *S*/*B* ratio, signal-to-background ratio; *S*/*N* ratio, signal-to-noise ratio; SD, standard deviation; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

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Materials and methods

Chemicals

2-Amino-2-methyl-1-propanol (AMP), pyridoxal-5′-phosphate hydrate (PLP), sodium pyruvate (Na pyruvate), L-KYN sulfate salt, zinc acetate (ZnOAc), sodium acetate (NaOAc), Tween 20, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), benzonase, HPLC-grade water and acetonitrile, L-glutamine, and Krebs–Ringer bicarbonate buffer (KRB buffer) were purchased from Sigma (St Louis, MO, USA).

huKAT I expression and purification

To generate KAT I enzyme for biochemical experiments, full-length human complementary DNA (cDNA), encoding an additional 6 histidine residues (His6) in frame at the C terminus, was expressed in insect cells and purified by nickel affinity chromatography essentially as described by Rossi and coworkers [13]. Briefly, insect Sf21 cells were infected with huKAT I-C-His6-carrying baculovirus and used to generate 10 L of cell suspension, from which cells were resuspended in 150 ml of lysis buffer (25 mM sodium phosphate buffer [pH 7.4], 0.1 mM PLP, 25 μg/ml benzonase, and 1 mM AEBSF), incubated on ice for 45 min, and lysed using microfluidizer twice. The lysate was centrifuged at 18,000*g* for 45 min, and the supernatant was loaded onto a nickel affinity column for His6 pull-down. The fractions containing huKAT I from the nickel affinity column were identified by Western blotting using anti-KAT I antibody (Genway, product no. 15-288-21989B) and then pooled, and the salt concentration of the pool was adjusted to 3 M NaCl. The pool was purified over a phenyl Sepharose column to eliminate impurities, and the huKAT I-containing fractions were concentrated and further purified on a size exclusion column (Superdex 200). The purified protein was concentrated to 1 mg/ml and flash frozen.

huKAT I in vitro activity assay

Reactions were performed in Costar black 96-well untreated plates (Fisher Scientific). According to final optimized conditions, 100 μl of reaction mixture contained 10 μl of compound or solvent (dimethyl sulfoxide [DMSO]), 0.3 ng/μl huKAT I, 90 μM l-KYN, 0.001% Tween 20, 1 μM PLP, and 1 mM Na pyruvate in 150 mM AMP buffer at pH 9.5. Compounds were preincubated with reaction components for 15 min before the addition of L-KYN and then were incubated for 4 h at 37 °C.

Measurement of KYNA using HPLC

Enzymatic reactions were quenched with 10 μl of 1 N HCl. Samples then were mixed with mobile phase (50 mM NaOAc and 5% acetonitrile, pH 6.2) at a 1:1 ratio, and 40 μl was injected into the column. The HPLC system consisted of a Waters 2795 Alliance separation module and a Waters 2475 multiwavelength detector (Milford, MA, USA) set at an excitation wavelength of 344 nm and an emission wavelength of 398 nm. The mobile phase was delivered at a flow rate of 1.5 ml/min, and 500 mM ZnOAc was delivered post-column by an external Shimadzu LC-20AD pump (Columbia, MD, USA) at a flow rate of 0.5 ml/min. The column was a Waters XBridge Shield RP18 column (3.5 μm, 4.6 × 50 mm). A retention time of 2.5–3.3 min for KYNA was observed under these conditions. KYNA peaks were processed using Waters Empower Analysis software. All measurements were performed in duplicates.

Measuring KYNA in a microplate

Here 100 μl of the buffer containing 50 mM NaOAc and 350 mM ZnOAc at pH 5.47 was added directly to each enzymatic reaction sample, and fluorescence was measured on a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA, USA). All measurements were done in duplicates. The signal-to-background (*S*/*B*) ratio, signal-to-noise (*S*/*N*) ratio, and *Z*′ factor for a 96-well plate format were calculated as follows: *S*/*B* ratio = (mean signal)/(mean background), *S*/*N* ratio = (mean signal − mean background)/standard deviation (SD) of background, and *Z*′ factor = [1 − 3 × (SDsignal + SDbackground)]/(mean of signal − mean of background) [14].

Generation of SH-SY5Y cell line stably expressing huKAT I

A plasmid-carrying huKAT I was purchased from Origene (product no. SC114349), and huKAT I was subcloned into Origene's pCMV-Neo plasmid via *Not*I restriction digestion. Sequence of the final product was confirmed by DNA sequence analysis (GENEWIZ). SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, and 1% penicillin/streptomycin. Cells (1×10^6) were transfected (2 μg of plasmid) by electroporation using an Amaxa nucleofector (Lonza, Switzerland) and were plated into 6-well plates. Then, 24 h after transfection, 400 μg/ml neomycin (Geneticin) was added to select for a pool of cells stably expressing huKAT I (7 days). The protein expression level was analyzed by Western blotting using anti-KAT I antibody (Genway, product no. 15-288-21989B).

huKAT I cell-based assay

SH-SY5Y cells stably expressing huKAT I were seeded in a 96-well collagen-coated plate and allowed to grow overnight. The following day, growth medium was removed and replaced with 100 μl of compound resuspended in KRB buffer (pH 7.0), 2% FBS, and 25 μM L-KYN. Plates were placed in an incubator (37 °C, 5% CO_2) overnight. Then 50 μl of the conditioned cell medium was transferred to a new plate containing 50 μl of 250 mM ZnOAc. Fluorescence was measured on a SpectraMax (Molecular Devices) or Envision (PerkinElmer) plate reader. HPLC measurements of KYNA in condition media were performed similarly to HPLC measurements of KYNA in in vitro enzymatic reactions as described above.

Results and discussion

Existing HPLC-based methods for the quantification of KYNA as a product of KAT enzymatic activity are based on chromatographic separation of KYNA on an HPLC column, followed by its detection based on either its electrochemical properties or the fluorescent properties as a complex with Zn^{2+} (λ_{ex} = 344 nm, λ_{em} = 398 nm) [15,16]. KYNA quantification by HPLC via fluorescence remains the more sensitive method, especially for measuring KYNA levels in brain and cerebrospinal fluid (CSF), where the levels of KYNA are in the low nanomolar (nM) range [3]. However, we observed that under the assay conditions for measuring KAT activity in vitro, where low micromolar (μM) amounts of KYNA are being formed, the only fluorescent species strongly emitting at 398 nm is the KYNA–Zn^{2+} complex (Fig. 1A and B). Hence, we recognized that chromatographic separation of the product is unnecessary and that fluorescence can be measured directly in the reaction mixture in the microplate.

Here, using KAT I as an example, we describe the development of a microplate fluorescence KAT assay for a 96-well format, although the assay is also suitable for miniaturization to 384- and 3456-well formats for HTS application. One of the criteria for HTS assay is to provide the best acceptable *S*/*B* ratio while using the minimal amount of reagents for which supply is limited (e.g.,