



An integrated experimental–computational platform for evaluating blood–brain barrier permeability of novel kynurenic acid derivatives

Zahraa Ali ^a, Bálint Lőrinczi ^b, Péter Simon ^b, Csilla Fazakas ^c, Imola Wilhelm ^c, Ferenc Bogár ^d, Linda Szilasi ^{a,e}, István Ilisz ^a, László Vécsei ^{f,g}, István Szatmári ^{b,h}, Róbert Berkecz ^{a,e,*}

^a Institute of Pharmaceutical Analysis, Faculty of Pharmacy, University of Szeged, Szeged, Hungary

^b Institute of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Szeged, Szeged, Hungary

^c Institute of Biophysics, HUN-REN Biological Research Centre, Szeged, Hungary

^d HUN-REN-SZTE Biomimetic Systems Research Group, Department of Medical Chemistry, University of Szeged, Szeged, Hungary

^e Department of Forensic Medicine, Albert Szent-Györgyi Health Centre, Szeged, Hungary

^f Department of Neurology, Albert Szent-Györgyi Faculty of Medicine, University of Szeged, Szeged, Hungary

^g HUN-REN-SZTE Neuroscience Research Group, University of Szeged, Szeged, Hungary

^h HUN-REN-SZTE Stereochemistry Research Group, University of Szeged, Szeged, Hungary

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ABSTRACT

Kynurenic acid (KYNA) is an endogenous neuroactive metabolite with considerable therapeutic potential, but its clinical translation is limited by poor blood–brain barrier (BBB) permeability. Although numerous KYNA derivatives have been developed to improve brain delivery, the molecular determinants governing BBB permeability remain insufficiently understood. Here, we established an integrated experimental–computational platform for the rational evaluation and optimisation of BBB permeability in novel KYNA derivatives. The platform combines a validated UHPLC–MS/MS assay, experimental determination of lipophilicity using a modified shake-flask method, and computational prediction of protonation states, tautomeric equilibria, solvent-accessible surface areas, and BBB-related molecular descriptors. Targeted structural modifications markedly improved lipophilicity and BBB permeability compared with KYNA. Apparent permeability coefficients (Papp) ranged from 3.4×10^{-6} to 1.1×10^{-5} cm s^{−1}, with **Compound 4a** exhibiting the highest permeability (3.23-fold higher than KYNA), followed by **Compounds 4b** (2.57-fold) and **4d** (2.11-fold). Experimental BBB permeability showed significant correlations with experimentally determined logD, predicted logBB, and solvent-accessible surface area descriptors. The superior permeability of **Compound 4a** was associated with a substantially higher population of the zwitterionic enol tautomer, suggesting a possible contribution of tautomeric equilibria to the observed permeability differences.

Overall, this work establishes an integrated platform linking molecular structure, physicochemical properties, and BBB permeability to support the rational evaluation and prioritisation of compounds within the investigated KYNA derivative series. Among the investigated derivatives, **Compound 4a** showed the most favourable *in vitro* BBB permeability profile and therefore warrants further pharmacokinetic and *in vivo* evaluation.

1. Introduction

Kynurenic acid (KYNA) is an endogenous neuroactive metabolite produced in the kynurenine pathway from the essential amino acid tryptophan (TRP). It modulates several neurotransmitter systems, including glutamatergic, cholinergic, dopaminergic, and GABAergic signalling, and exerts neuroprotective and anti-excitotoxic effects

through antagonism of *N*-methyl-D-aspartate and other glutamate receptors, as well as free radical scavenging activity (Alves et al., 2024; Grishanova and Perepechaeva, 2024; Molnár et al., 2021; Vécsei et al., 2013). Altered KYNA levels have been associated with numerous neurological, neurodegenerative, inflammatory, and metabolic disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease, schizophrenia, multiple sclerosis, diabetes, and COVID-19,

* Corresponding author.

E-mail address: berkecz.robert@szte.hu (R. Berkecz).

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highlighting its potential as both a therapeutic target and biomarker (Alves et al., 2024; Cavaleri et al., 2024; Fathi et al., 2022; Ji et al., 2017; Vécsei et al., 2013; Walczak et al., 2020). Despite its broad therapeutic potential, the clinical application of KYNA is severely limited by its poor pharmacokinetic properties, particularly its low blood–brain barrier (BBB) permeability following systemic administration. Therefore, considerable efforts have been devoted to the development of KYNA derivatives with improved BBB penetration while preserving the favourable pharmacological properties of the parent compound. Among these, **Compound 1** and **Compound 2**, previously developed by our group, demonstrated enhanced BBB permeability and promising neuroprotective effects (Demeter et al., 2012; Domoki et al., 2023; Kovács et al., 2021; Molnár et al., 2021). **Compound 1** exhibited increased brain bioavailability and improved neuronal activity in a translational model of hypoxic–ischemic encephalopathy, and has also shown therapeutic potential in migraine, acute necrotising pancreatitis, and sepsis (Balla et al., 2021; Domoki et al., 2023; Juhász et al., 2020; Knyihár-Csillik et al., 2008; Kovács et al., 2021; Németh et al., 2004). Likewise, **Compound 2** reduced hippocampal population spike activity and protected against pentylentetrazol-induced epileptiform seizures, findings that were further supported by *in vitro* studies (Demeter et al., 2012; Németh et al., 2004).

From an analytical standpoint, the determination of KYNA, structurally diverse KYNA derivatives, and other TRP metabolites in biological samples remains challenging because of their wide range of polarity, ionisation behaviour, and concentration levels (Poojary et al., 2021). Various analytical approaches, including HPLC coupled with photodiode array (DAD), fluorescence (FD), and tandem mass spectrometry (MS/MS), have been reported for TRP metabolite analysis (Arnhard et al., 2018; Du et al., 2018; Fukushima et al., 2007a; Fukushima et al., 2007b; Nadour et al., 2022; Pedraz-Petrozzi et al., 2023; Sadok et al., 2020; Sousa et al., 2021; Tong et al., 2018; Zhao et al., 2010). While HPLC-FD requires pre- or post-column derivatisation for KYNA detection (Du et al., 2018; Fukushima et al., 2007b; Sousa et al., 2021), UHPLC-MS/MS and particularly UHPLC-MS/HRMS have become the methods of choice owing to their superior selectivity and sensitivity (Arnhard et al., 2018; Gáspár et al., 2022; Nadour et al., 2022; Pedraz-Petrozzi et al., 2023; Tong et al., 2018). However, only a limited number of studies have addressed the determination of KYNA derivatives in biological matrices (Domoki et al., 2023; Kovács et al., 2021; Molnár et al., 2021). In our previous work, targeted UHPLC-MS/HRMS confirmed the *in vivo* BBB permeability and brain distribution of **Compound 1** (Domoki et al., 2023; Kovács et al., 2021). Accordingly, **Compound 1**, **Compound 2**, KYNA, and newly synthesised KYNA derivatives were evaluated using an *in vitro* BBB model. To support quantitative permeability assessment, a sensitive and selective UHPLC-MS/MS method was developed and validated. This is the first report describing the simultaneous determination of these novel KYNA derivatives.

Lipophilicity is a key determinant of drug absorption, membrane permeability, tissue distribution, including BBB penetration, and elimination. It is commonly characterised by the octanol/water partition coefficient (logP), whereas the distribution coefficient (logD) describes the partitioning of ionizable compounds at a defined pH and therefore provides a more physiologically relevant measure of lipophilicity. Accordingly, logD is typically determined at physiological pH (7.4) (Arnott and Planey, 2012; Hill and Young, 2010; Organisation for Economic Co-operation and Development, 1995; Organisation for Economic Co-operation and Development, 2022). To complement the BBB permeability studies, we experimentally determined the logP and logD values of KYNA and its derivatives using a modified shake-flask approach. The modified procedure was designed to improve experimental robustness and throughput by combining controlled phase partitioning with simultaneous UHPLC-MS/MS quantification of multiple analytes, using internal standards for reliable quantitative analysis, and maintaining a controlled temperature throughout the experiment. In

addition, DMSO was avoided as a cosolvent to minimise potential perturbation of analyte distribution between the aqueous and organic phases. We further correlated the experimental lipophilicity data with computational descriptors and *in vitro* BBB permeability to establish an integrated structure–property–permeability framework. However, BBB permeability cannot be reliably explained by lipophilicity alone, because membrane transport reflects the combined influence of multiple molecular properties, including ionisation state, tautomeric equilibria, molecular size, and solvent-accessible surface area. Therefore, evaluating permeability or individual physicochemical descriptors in isolation provides limited information on the structural determinants governing BBB penetration. Integrating experimental permeability and lipophilicity measurements with computational characterisation may provide a more comprehensive structure–property–permeability relationship, enabling the identification of molecular features associated with improved BBB transport and supporting the rational prioritisation of KYNA derivatives for further investigation.

Therefore, the aim of this study was not only to quantify the BBB permeability of novel KYNA derivatives but also to establish an integrated experimental–computational framework linking molecular structure, physicochemical properties, and BBB permeability to support rational lead optimisation. To achieve this, we combined a validated UHPLC-MS/MS assay for quantitative analysis, experimental determination of lipophilicity using a modified shake-flask approach, *in vitro* BBB permeability measurements, and computational prediction of protonation states, tautomeric equilibria, and BBB-related molecular descriptors. This integrated platform was applied to identify structural determinants governing BBB permeability and to prioritise promising KYNA derivatives for further preclinical evaluation.

2. Materials and methods

2.1. Chemicals and standards

LC-MS grade acetonitrile (ACN), water (H₂O), methanol (MeOH), 2-propanol (IPA), formic acid (FA), and dichloromethane (DCM, reagent purity) were provided by VWR (Radnor, PA, USA). Ammonia (NH₃) 32%, ascorbic Acid, saline solution, Ringer-HEPES buffer, 1-octanol (99%, Thermo Scientific). Ammonium sulfate from Brenntag, Hungary. Standards of the following compounds were synthesized and provided by the Institute of Pharmaceutical Chemistry, University of Szeged (Table 1). Each standard compound was dissolved in MeOH, except KYNA, which was dissolved in DMSO, obtaining a 1 mg/mL solution. For further dilutions, MeOH was used.

Compounds 1–3 and **ISTD 1**, **Compounds 4b** and **4c**, and **ISTD 2** were synthesised according to the literature (Fehér et al., 2019; Lőrinczi et al., 2020; Sárík et al., 2024). The synthesis of the newly investigated derivatives, **Compounds 4a** and **4d**, as well as the synthetic intermediates **Compounds 5** and **6**, is detailed in Section S1 of the Supplementary Information, together with the corresponding NMR data. **Compounds 5** and **6** served as synthetic intermediates and were therefore not included in the subsequent physicochemical characterisation and *in vitro* BBB permeability experiment.

2.2. Permeability assay

The *in vitro* BBB experiments were performed using our previously published methodology (Molnár et al., 2021). Briefly, the *in vitro* blood–brain barrier model was constructed comprising rat brain endothelial cells cultured on semipermeable filter inserts, having rat brain pericytes on their bottom surface and rat astrocyte-conditioned media in the lower chambers. Upon reaching confluence, the endothelial monolayer was supplemented with 550 nM hydrocortisone, 250 μ M CPT-cAMP, and 17.5 μ M Ro-20-1724 (all from Merck/MilliporeSigma) to induce maturation of tight junctions. After the transendothelial electric resistance reached plateau of 150–170 $\Omega \times \text{cm}^2$, cells were

Table 1
Structures and IUPAC names of investigated KYNA and its derivatives.

Name	IUPAC name	Structure
ISTD 1	<i>N</i> -(3-(dimethylamino)propyl)-4-oxo-1,4-dihydroquinoline-2-carboxamide	
ISTD 2	3-(morpholinomethyl)-4-oxo-1,4-dihydroquinoline-2-carboxamide	
KYNA	4-oxo-1,4-dihydroquinoline-2-carboxylic acid	
Compound 1	<i>N</i> -(2-(dimethylamino)ethyl)-4-oxo-1,4-dihydroquinoline-2-carboxamide	
Compound 2	<i>N</i> -(2-(dimethylamino)ethyl)-3-(morpholinomethyl)-4-oxo-1,4-dihydroquinoline-2-carboxamide	
Compound 3	3-(morpholinomethyl)-4-oxo- <i>N</i> -(2-(pyrrolidin-1-yl)ethyl)-1,4-dihydroquinoline-2-carboxamide	
Compound 4a	8-chloro- <i>N</i> -(2-(dimethylamino)ethyl)-3-(morpholinomethyl)-4-oxo-1,4-dihydroquinoline-2-carboxamide	
Compound 4b	7-chloro- <i>N</i> -(2-(dimethylamino)ethyl)-3-(morpholinomethyl)-4-oxo-1,4-dihydroquinoline-2-carboxamide	

Table 1 (continued)

Name	IUPAC name	Structure
Compound 4c	5-chloro- <i>N</i> -(2-(dimethylamino)ethyl)-3-(morpholinomethyl)-4-oxo-1,4-dihydroquinoline-2-carboxamide	
Compound 4d	6-chloro- <i>N</i> -(2-(dimethylamino)ethyl)-3-(morpholinomethyl)-4-oxo-1,4-dihydroquinoline-2-carboxamide	

washed in Ringer-HEPES. The compounds were applied to the top chamber at a final concentration of 10 μM . After 60 min, samples were removed from the bottom compartments and kept at -80°C until analysis.

The apparent permeability coefficient (P_{app}) was determined by the following equation: $P_{\text{app}} = \Delta Q / (\Delta t \times 60 \times A \times C_0)$ (cm/s) where $\Delta Q / \Delta t$ is the permeability rate ($\mu\text{g}/\text{min}$), C_0 is the initial concentration or the concentration in the donor chamber ($\mu\text{g}/\text{cm}^3$), and A is the membrane surface area (0.33 cm^2) (Artursson, 1990).

Dedicated cytotoxicity or cell-viability assays were not performed for the investigated compounds under the conditions used in the present permeability experiments; therefore, this aspect should be considered a limitation of the current study.

2.3. Sample preparation procedure for targeted UHPLC–MS/MS measurements

Initially, 50 μL from each *in vitro* sample (400 μL in total for the eight compounds) was pooled in a microcentrifuge tube. Ringer–HEPES buffer (200 μL) was then added, followed by vortex mixing. From the resulting diluted solution, 120 μL was taken forward for sample preparation. The *in vitro* samples or the matrix-matched calibration samples were prepared by placing 150 mg of ammonium sulfate with 10 μL 100 pmol/mL of ISTD 1 methanolic solution, spiked 120 μL of the pooled *in vitro* samples (or 120 μL Ringer-HEPES buffer for calibration samples), and 20 μL of MeOH (or 20 μL of the given calibration mixture for calibration). Then, 500 μL of the precipitation solution (5 mM EDTA, 2.5 mM ascorbic acid in physiological solution/MeOH) at 1/10 (v/v%) was added, followed by vortexing and ultrasonication for 5 min. For sample centrifugation, 15,000 rpm was applied for 15 min at 4°C . The resulting supernatant (500 μL) was collected and evaporated to dryness under a gentle stream of nitrogen. The dried extract was dissolved in 50 μL of the dissolving solution ($\text{H}_2\text{O}/\text{MeOH}/\text{NH}_3$ (4%)) (90/10/0.1 v/v/v%), then centrifuged at 15,000 rpm for 5 min at 25°C . The upper phase was transferred to a conical insert and measured by UHPLC–MS/MS. The following calibration points were used: 0, 5, 10, 20, 50, 100, 150, 200, and 400 pmol/mL sample (Fig S7).

2.4. Flow injection - HESI-MS/HRMS parameters of fragmentation study

The fragmentation behavior of targeted analytes was studied using a Thermo Scientific Exploris 240 Mass Spectrometer (Waltham, MA, USA) operated with heated electrospray ionization (HESI) mode coupled to a Waters Acquity I-Class UPLC (Milford, MA, USA). MassLynx 4.1 (Milford, MA, USA) was used to control the UHPLC instrument, and HRMS data were acquired using Xcalibur 4.4 (Waltham, MA, USA). The flow

injection method was used by introducing the H₂O/ACN/FA (30/70/0.1, v/v/v%) mobile phase delivered by a UHPLC pump and combined with the solution of the given compound (20 µM) carried with a syringe pump at a flow rate of 0.1 mL/min and 0.01 mL/min into the HESI source via a mixing tee.

HESI parameters were set as follows: spray voltage set to 4.5 kV in positive ionization mode, the temperature of ion transfer tube and vaporizer gas were set to 300 °C, the vaporizer temperature set to 300 °C, the sheath gas flow set to 25, the sweep gas flow set to 5, and the auxiliary gas flow set to 0 in arbitrary units. For the full HRMS measurement, the resolution was 60 000, the scan range was between 150–500 *m/z*, and the S-Lens RF level was 70. The AGC target was set to 10⁶, and the maximum injection time was 100 ms. For the MS/HRMS, generally, the isolation windows of precursor ions were set to 1 *m/z*, while 5 *m/z* was used for **Compounds 4a, 4b, 4c, and 4d** to detect the chlorine isotope peaks of fragment ions. The following HCD collision energy values were used 10, 20, 30, 40, 50, and 60 eV, respectively. The resolution was set to 15 000, the S-Lens RF level to 70, the AGC target to 10⁶, and the maximum injection time to 100 ms.

2.5. Targeted UHPLC–MS/MS parameters for quantitative analysis

For targeted UHPLC–MS/MS analysis, a Shimadzu Nexera ultra-high-performance liquid chromatography (UHPLC) system (Kyoto, Japan) was used, connected to the TSQ Fortis triple quadrupole tandem mass spectrometer (Thermo Scientific, Waltham, MA, USA) via a 2-position, 6-port valve. The Agilent 1100 HPLC pump (Agilent, Santa Clara, CA, USA) served as the auxiliary pump, delivering the HESI source-rinsing solution (H₂O/ACN, 10/90, v/v%). LabSolution Version 5.97 SP1 (Shimadzu, Kyoto, Japan) was used to control the UHPLC system, while Xcalibur 4.2 software (Thermo Fisher Scientific, Waltham, MA, USA) was used for the data acquisition and the quantitative evaluation of MS/MS raw files.

The reversed-phase column (ACQUITY UPLC HSS T3 C18, 100 × 2.1 mm, 1.8 µm, 100 Å, Waters) was fitted with a guard column packed with the same stationary phase. (Milford, MA, USA) was used to separate targeted analytes. The column was thermostated at 30 °C during analysis, and a flow rate of 0.4 mL/min was used. A gradient elution was applied using eluent A (1 v% FA in 5 mM ammonium formate solution), and eluent B consisting of 1 v% FA in 5 mM ammonium formate containing ACN, respectively. The final gradient program was as follows: 0-1-5-5-5-6.5-7-10 min 5-5-40-100-100-5-5% B.

The injected volume was 10 µL, and the ACN/H₂O/FA (60/40/0.1, v/v/v%) solution was used for washing the injector needle after each injection. To minimize contamination of the mass spectrometer, the UHPLC effluent was directed to the ESI source only between 1 and 5 min using an automatically controlled 2-position, 6-port divert valve. During the remaining time, a source-rinsing solution was delivered to the ESI source via the auxiliary pump at a flow rate of 0.3 mL/min to prevent contamination of the source. Total run time was 10 min.

The ESI source parameters used in the final UHPLC–MS/MS method were as follows: the ion transfer tube temperature was set to 300 °C, the vaporizer temperature to 350 °C, the spray voltage to 4 kV, the sheath gas flow to 20 arbitrary units (a.u.), the auxiliary gas flow to 1 a.u., and the sweep gas flow to 5 a.u. KYNA derivatives were detected in a multiple reaction monitoring (MRM) in the positive ionization mode with 0.7 resolution for Q1 and Q3 quadrupoles. Collision-induced dissociation (CID) was performed using argon as the collision gas at a pressure of 1.5 Pa. The source and MS/MS parameters were optimized for the quantifier and qualifier ions of the protonated targeted analytes precursor ions via the flow injection method as follows: H₂O/ACN/FA (30/70/0.1 v/v/v%) mobile phase delivered by a UHPLC pump and merged with the solution of the given compound (20 nmol/mL) introduced with Syringe pump at of 0.1 mL/min and 0.01 mL/min flow rate of 0.1 mL/min and 0.01 mL/min into the ESI source via mixing tee. MS/MS parameters of five fragment ions of each targeted compound were

optimized, and the two most intense fragment ions were selected as quantifier ion (for quantitation) and qualifier ion (for confirmation). The main UHPLC–MS/MS parameters are summarized in [Table 2](#).

2.6. Sample preparation procedure for logP and logD determination via modified shake-flask approach

2.6.1. Pre-saturation of the aqueous and organic phase

For logP determination, water-saturated octanol and octanol-saturated water were generated (logP equilibrium solution) by vigorous mixing of equal volumes of MS-grade water and MS-grade 1-octanol, followed by vigorous shaking, then leaving to rest for 24 h at 25 °C until complete separation. Similarly, a pre-saturated mixture of Ringer-HEPES buffer/octanol (logD equilibrium solution) was prepared for logD determination by mixing equal volumes of 1-octanol with Ringer-HEPES buffer (same buffer of the biological assay, pH=7.4) followed by a 24 h rest at room temperature to achieve full phase separation.

2.6.2. Standards solutions for logP and logD experiments

Standards of the eight KYNA derivatives ([Table 1](#)) were prepared at 100 nmol/mL in MeOH, and 40 µL of each standard was pipetted into two microcentrifuge tubes (the first tube was the equilibrium sample, and the second was the initial solution sample). The resulting sixteen microcentrifuge tubes (Costar, USA) (eight were equilibrium samples and the other eight were initial solution samples) were evaporated under nitrogen. An internal standard of the newly synthesized KYNA derivatives (**ISTD 2**, [Table 1](#)) was used to minimize the standard deviation of quantitative data arising from variations in sample preparation and other analytical steps. A stock solution of the internal standard (**ISTD 2**) at 100 nmol/mL in MeOH was prepared and then diluted to 2.5 nmol/mL in MeOH.

A standard mixture of KYNA and its derivatives was prepared with a concentration of 20 nmol/mL, and it was used to prepare the calibration series with the concentration points (0, 0.1, 0.4, 0.8, 1, 2 nmol/mL) with H₂O/MeOH/4% NH₃ (90/10/0.1 v/v/v%) solution.

2.6.3. Optimised procedure of logD and logP determination

From the pre-saturated mixture of octanol/Ringer-HEPES-buffer (logD equilibrium solution), equal volumes of 700 µL of octanol and 700 µL of Ringer-HEPES-buffer were pipetted into the microcentrifuge tube, which contains an evaporated standard of KYNA derivative as mentioned in the previous paragraph, and then were vortexed, ultrasonicated, and centrifuged (500 rpm, 25 °C, 15 min) to separate the organic and the buffer phase. Each buffer phase was collected in a separate vial. The pooled buffer sample was prepared by combining 10 µL of each of the eight buffer phases with 10 µL of the 2.5 µM **ISTD 2** solution, yielding a final volume of 90 µL and a 9-fold dilution of each buffer solution, ready for UHPLC–MS/MS measurement.

To the initial solution samples (the other eight microcentrifuge tubes containing the given evaporated analytes), 1400 µL of solvent mixture (H₂O/MeOH/4% NH₃ (90/10/0.1 v/v/v%)) was added and vortexed (**Fig S8**). For UHPLC–MS/MS measurement, the pooled sample of initial solution samples was prepared by mixing of 10 µL of each of the initial solutions and 10 µL of the 2.5 nmol/mL **ISTD 2** solution, obtaining 90 µL of final volume.

For logP determination, the same procedure was applied, only water was used instead of Ringer-HEPES buffer.

Three replicates were prepared over three days for comparison. All pooled samples were analyzed using the developed UHPLC–MS/MS method; only the autosampler temperature was set to 25 °C rather than 4 °C (**Fig S8**).

2.7. Validation of the targeted UHPLC–MS/MS method

To support the quantitative application of the developed targeted

Table 2

Targeted UHPLC–MS/MS main parameters of investigated analytes.

Compound	Type of ion	Retention time (min)	Precursor (m/z)	Product (m/z)	Collision energy (eV)	Tube Lens (V)
ISTD 1	quantifier ion	4.22	274	229	17	65
	qualifier ion	4.22	274	144	37	65
ISTD 2	quantifier ion	2.85	288	201	18	65
	qualifier ion	2.85	288	173	24	65
KYNA	quantifier ion	4.35	190	144	18	65
	qualifier ion	4.35	190	89	39	65
1	quantifier ion	4.02	260	215	17	65
	qualifier ion	4.02	260	144	31	65
2	quantifier ion	1.36	359	272	15	65
	qualifier ion	1.36	359	227	23	65
3	quantifier ion	1.78	385	298	14	65
	qualifier ion	1.78	385	227	28	65
4 (a,b,c,d)	quantifier ion	3.00	393	306	15	65
	qualifier ion	3.00	393	261	25	65

UHPLC–MS/MS method, a bioanalytical validation was performed in accordance with the ICH M10 Guideline 2022 in terms of matrix effect, linearity range of the calibration curve, lower limit of quantification (LLOQ) to upper limit of quantification (ULOQ), carry-over, precision, accuracy, stability and reinjection reproducibility (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), 2022). A detailed description of the validation procedure is provided in the S2 section of the Supplementary Information.

2.8. Data processing

Qualitative information was obtained by manual inspection of the UHPLC–MS/MS and flow injection-HRMS/MS raw data, whereas quantitative evaluation was carried out using the integrated processing workflow implemented in Xcalibur 4.2 software. The statistical evaluations and association between variables were performed in GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) using Spearman's rank correlation and ANOVA. The algorithm figures were generated with BioRender.com.

ROWAN workflows were used to generate predicted logD, logP, and microscopic pKa values of KYNA derivatives (Nevolianis et al., 2025; Swanson et al., 2023; Wagen and Wagen, 2024), while the ChemAxon logD calculator (web tool) was used to obtain predicted logD values (ChemAxon, 2024). Predicted logBB was calculated from the compound structure using the way2drug.com BBB permeability (GEB) web server (Way2Drug, 2024).

2.9. Calculation of macro pKa and polar surface area (PSA)

Macroscopic pKa values were predicted using the Epik-ML method (Johnston et al., 2023) implemented in Schrödinger's software suite (Schrödinger, 2025). Epik-ML employs a machine learning-enhanced approach that builds on prior Epik versions by combining rapid empirical predictions with physics-based corrections derived from density functional theory calculations. This method first enumerates all relevant protonation states and tautomers of the molecule, then computes micro-pKa values for individual titratable sites using a linear free-energy relationship perturbed by chemical fragments and charge spreading. Macro-pKa values are subsequently determined from micro-pKa values by solving thermodynamic equilibrium equations that account for interactions between sites. From the macroscopic pKa values the relative population of tautomers with different charge states were also calculated at a physiological pH. The total solvent-accessible surface areas of the investigated compounds were calculated using the atomic.sasa.py Python utility from the Schrödinger's software package (Schrödinger, 2025).

3. Results and discussion

3.1. UHPLC–MS/MS method development and validation

3.1.1. Study of mass spectrometric behaviour of KYNA and its derivatives

Investigation of ESI polarity showed that positive ionisation provided markedly higher ionisation efficiency than negative mode because of the amidation of the KYNA carboxyl group and the presence of tertiary amines in the derivatives. Therefore, positive ionisation was selected for MS/HRMS and MS/MS fragmentation studies and detailed in the Supplementary Information (Section S3). The oxo tautomer of KYNA was confirmed to be the predominant form, consistent with previous reports (Lőrinczi et al., 2020; Schmidt et al., 2024; Zelentsova et al., 2013). Although KYNA has been extensively investigated, its detailed fragmentation pathway has not previously been described. The proposed fragmentation pathway is presented in Fig. S9.

Comparison of MS/HRMS and MS/MS spectra revealed notable differences. In particular, the fragment ion at m/z 162.05496, one of the most abundant ions in the HRMS spectrum, showed only low abundance in MS/MS experiments, most likely because of the different collision gases used (nitrogen versus argon). Accordingly, the fragment ions at m/z 144 and 89 were selected as the quantifier and qualifier ions for quantitative LC–MS/MS analysis.

Compounds 1 and ISTD 1 exhibited closely related fragmentation pathways differing by a single methylene group, whereas **Compound 3** followed a similar fragmentation mechanism with formation of the characteristic m/z 98.09643 fragment (Figs. S10–S11). In contrast, **Compounds 2 and 4a–d** displayed distinct fragmentation behaviour owing to the morpholinomethyl substituent, which promoted fragmentation through loss of the morpholine moiety (Fig. 1; Figs. S12–S16). Monochloro-substituted derivatives fragmented similarly to **Compound 2**, and no substantial differences were observed among the positional isomers despite the different chlorine substitution patterns. **Compound 3** also followed the same initial α -cleavage mechanism as the other derivatives despite replacement of the dimethylamino group by a pyrrolidine ring (Fig. 1; Figs. S10, S12–S16). The optimised MRM transitions and collision energies used for quantitative LC–MS/MS analysis are summarised in Table 2.

3.1.2. UHPLC method development for the target compounds

Chromatographic development focused on achieving baseline separation of all KYNA derivatives within a short analysis time while maintaining satisfactory peak shape, particularly for KYNA, which exhibited peak broadening under suboptimal conditions. Based on our previous experience with KYNA analysis, the ACQUITY UPLC HSS T3 C18 column was selected for method development.

Initial experiments using methanol as the organic modifier provided satisfactory separation for most analytes; however, positional isomers **Compounds 4b and 4d** co-eluted (Fig. S17). Systematic optimisation of

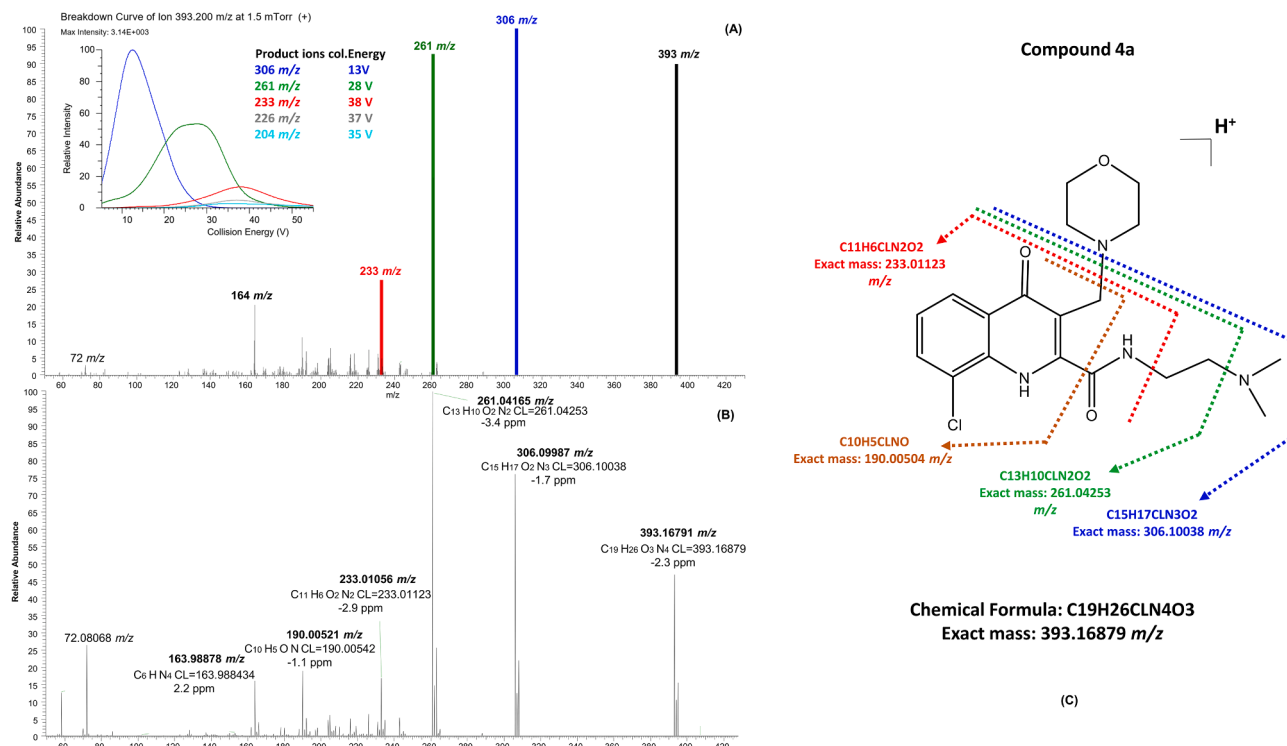


Fig. 1. MS/MS spectrum of **Compound 4a** with related CE optimisation (A), MS/HRMS spectrum of **Compound 4a** (B) (CE, 10–60 eV), proposed fragmentation pathway of **Compound 4a** (C).

chromatographic parameters, including column temperature, gradient profile, and initial mobile-phase composition, did not achieve baseline separation. Replacing methanol with acetonitrile and supplementing both mobile phases with 5 mM ammonium formate markedly improved selectivity, enabling complete separation of all targeted analytes within 5 min (Fig. 2 and Fig. S17).

The optimised UHPLC–MS/MS method yielded sharp, symmetrical peaks for all compounds and was subsequently validated in accordance with the ICH M10 guideline. The validation parameters met all pre-defined acceptance criteria, confirming the method's suitability for reliable quantification of KYNA derivatives in the *in vitro* BBB permeability study. A detailed description of chromatographic optimisation and validation is provided in the Supplementary Information (Section S3–4).

3.2. LogP and logD values obtained using a novel shake-flask method

Experimental determination of logP and logD provides valuable physicochemical descriptors that complement BBB permeability studies. Although predicted values are widely available, experimental data remain scarce for KYNA and its derivatives, particularly for structurally related positional isomers (ChemAxon, 2024; Hill and Young, 2010; Nevolianis et al., 2025). Therefore, experimental logP and logD values were determined using a modified shake-flask approach, as detailed in Section S5. The method was optimised using nimodipine (NMD) as a reference compound (Barbato et al., 1996). Several modifications were introduced to improve accuracy and experimental throughput, including temperature-controlled partitioning and centrifugation, simultaneous UHPLC–MS/MS quantification of pooled aqueous phases, indirect calculation of analyte concentrations in the organic phase from

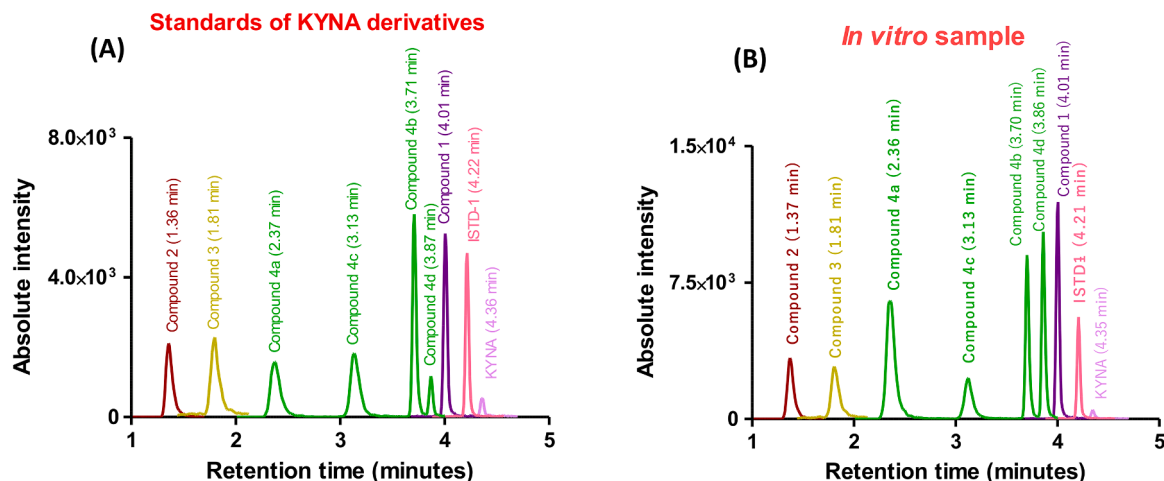


Fig. 2. The extracted ion chromatograms of KYNA derivatives in a standard mixture (A), and *in vitro* sample (B).

mass balance, and omission of DMSO as a cosolvent to avoid perturbation of phase distribution (Alelyunas et al., 2010; Ford et al., 1991; Low et al., 2016; Wilson et al., 2001). The experimentally determined logD value of NMD (4.00–4.19) was in excellent agreement with the reported literature value (4.18) (Barbato et al., 1996), thereby validating the applicability of the developed methodology.

The logP and logD values of the investigated compounds were determined using our newly developed approach and exhibited low standard deviations. The logP and logD ranges of the compounds were within ± 0.3 range, consistent with OECD guideline recommendations. Furthermore, the experimentally obtained values were compared with predicted values to assess their agreement (Nevolianis et al., 2025; Swanson et al., 2023; Wagen and Wagen, 2024). All investigated analytes exhibited negative logP values (Table 3); the lowest was observed for KYNA (−1.17), and the highest for the 4b isomer (−0.26). The low value observed for KYNA, compared with its derivatives, can be attributed to the presence of a free carboxyl group and the lack of a heterocyclic and aliphatic tertiary amine chain. In the case of Compounds 4c and 4d, where the chloro substituent occupies the 5- and 6-positions, comparable logP values were obtained. In contrast, the 8- and 7-substituted constitutional isomers of 4a and 4b exhibited markedly different logP values. Interestingly, all logP values calculated using the Rowan platform were positive (Table 3). The lowest predicted value was observed for Compound 2 (1.06). In contrast, the chloro-substituted isomers consistently exhibited high logP values (1.72), indicating that the position of the chloro substituent does not affect the lipophilicity of the analyte. The substantial differences between the experimental and predicted logP values may be related to the ionizable nature of the investigated KYNA derivatives and their ability to adopt different protonation and tautomeric states. Experimental logP values reflect the partitioning behaviour under the specific octanol–water conditions applied, whereas the calculated values are model-dependent estimates whose accuracy may depend on the representation of ionisation, tautomerism, and the chemical space covered by the prediction model. These factors may contribute to the observed discrepancies, indicating that the predicted logP values should be interpreted with caution and regarded as complementary to experimentally determined lipophilicity.

Since logD is determined at a given pH, it corresponds to the value used during treatment, thereby providing more accurate information about the lipophilicity of the analytes under study than logP values. Furthermore, measurements performed in buffered solutions suppress the compound's ability to alter the pH of the aqueous phase, whereas pure water is not buffered and thus allows the compound to do so. Therefore, in our work, we focused on logD values obtained in a buffer solution (pH 7.4) used for the *in vitro* BBB permeability assays, and the calculated logD values are summarised in Table 4. As with logP values, the lowest logD value was observed for KYNA; however, all other compounds exhibited positive logD values. As observed for the logP values, the lowest logD values (among the four isomers) were obtained for 4a isomer, which were comparable to those of the 4c isomer. For Compounds 4b and 4d, similar logD values were observed. When

comparing the calculated and experimentally determined logD values, the Rowan-predicted values for KYNA were very close and correlated with the experimental values (Fig. S18), and a similar level of agreement was observed for Compounds 3, 4b, and 4d. For Compounds 1, 4a, and 4c, the ChemAxon predictions provided values closer to the experimentally measured results and the correlation is shown in Figure S18. Upon examining the relationship between the Rowan- and ChemAxon-predicted logD values, a stronger correlation was observed with the experimental values for all compounds [Fig. S18, S19]. As a further confirmation, we investigated the relationship between the retention times obtained from the reversed-phase measurements and the logD values, with the caveat that the eluent pH during the chromatographic measurements was 2.32 (5 v/v% B), 2.39 (20 v/v% B), and 2.69 (40 v/v%), in contrast to the pH 7.4 used for logD determination. It is important to note that these pH values are only approximate due to the presence of an organic solvent. For Compounds 2, 3, and 4a–d, a Spearman's correlation coefficient of 0.94 was obtained, which can be considered to be a strong relation (Figure S20). In contrast, KYNA and Compound 1 showed outlying values, which can be explained by the absence of a morpholinomethyl group in these two compounds. At the applied eluent pH, this group is protonated and significantly influences the retention times of the corresponding derivatives. The pKa of KYNA is 2.43, and it slightly increases in the present organic solution. At the applied pH, the ratio of the carboxyl groups to the carboxylate ions is comparable, resulting in higher logD values than at pH 7.4, where only the deprotonated form is present (Buzásy et al., 2025). Overall, the structural modification of KYNA increased the lipophilicity of its derivatives, as supported by both experimental and predicted logP/logD values.

3.3. Relationship between the *in vitro* permeability and structural characteristics of the investigated analytes

Using an *in vitro* model system employed in this study, the permeability of KYNA and its previously known and newly synthesised derivatives was investigated. This *in vitro* model reliably mimics the *in vivo* blood–brain barrier permeability characteristics of the targeted compounds, and it is suitable for preliminary screening prior to further *in vivo* investigations. The applicability of this model to KYNA derivatives is well demonstrated by our previous *in vitro* and *in vivo* studies, in which *in vitro* investigations of Compound 2 revealed relatively high permeability, and *in vivo* experiments of Compound 1 confirmed its permeability in neonatal piglets and mice models (Balog, 2024; Balog et al., 2025; Domoki et al., 2023; Kovács et al., 2021; Martos et al., 2025; Molnár et al., 2021). Therefore, during the investigation of the novel derivatives, we considered it important to examine, in addition to KYNA as a low-permeability reference compound, Compounds 1 and 2, which have relatively higher permeability, as reference substances. In the case of Compound 1, amidation of the KYNA carboxyl group with a dialkylaminoethyl moiety markedly increased its lipophilicity, as confirmed by our logD results, thereby facilitating its penetration across the blood–brain barrier. Our investigations were extended to evaluate how structural modifications to the quinoline ring and the aliphatic-cyclic tertiary amine group influence the *in vitro* penetration properties of the compounds.

Initial concentrations (C_0) of the studied compounds and their concentrations (dQ) in the solutions collected beneath the BBB-forming cerebral endothelial cells during the *in vitro* experiments were quantified using pooled samples, and the P_{app} values are summarized in Table S2. The obtained P_{app} values ranged from 3.4×10^{-6} to 1.1×10^{-5} cm/s, with the lowest value observed for KYNA and the highest for the 4a isomer. The P_{app} of KYNA is located in low- to moderate-permeability range. In contrast, the corresponding derivatives predominantly exhibit moderate permeability, with Compounds 4a, 4b, 4c, and 4d showing particularly favorable permeability values (Kus et al., 2023). For clearer interpretation, permeability data were

Table 3
Experimental and calculated logP values.

Compound	Experimental logP values	Experimental logP range	Predicted logP (via ROWAN platform)
KYNA	−1.17	(−1.29 to −1.03)	1.64
1	−1.02	(−1.19 to −0.92)	1.23
2	−0.72	(−0.82 to −0.73)	1.06
3	−0.92	(−1.26 to −0.88)	1.60
4a	−0.9	(−1.19 to −0.75)	1.72
4b	−0.26	(−0.24 to −0.23)	1.72
4c	−0.45	(−0.63 to −0.33)	1.72
4d	−0.44	(−0.56 to −0.38)	1.72

SD: standard deviation.

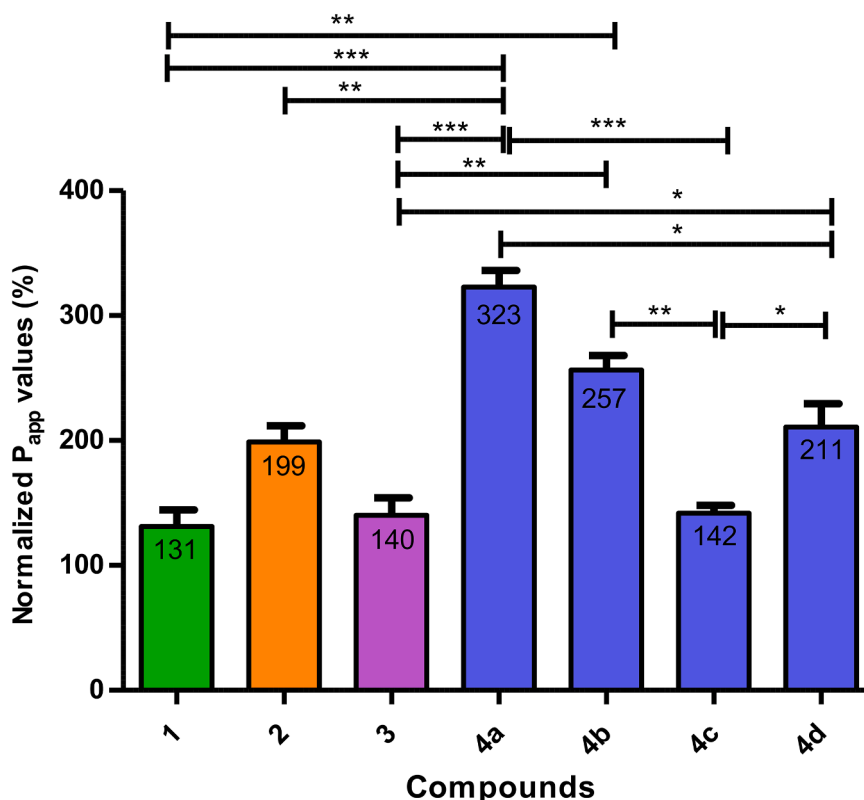
Table 4

Experimental and predicted logD and logBB values.

Compound	Experimental logD values (pH=7.4)	Experimental logD range (pH=7.4)	Predicted logD (pH=7.4) (Chemaxon)	Predicted logD (pH=7.4) (ROWAN)	Predicted logBB (way2drug)
KYNA	-1.04	(-1.36 – -0.93)	-1.21	-1.00	-0.67
1	0.27	(0.26 – 0.31)	0.17	-0.30	-0.47
2	0.28	(0.22 – 0.35)	-0.18	-0.40	-0.53
3	0.29	(0.17 – 0.36)	0.04	0.21	-0.53
4a	0.35	(0.26 – 0.40)	0.45	0.69	-0.32
4b	0.92	(0.87 – 0.93)	0.44	0.79	-0.26
4c	0.49	(0.49 – 0.52)	0.53	0.59	-0.21
4d	0.88	(0.82 – 0.93)	0.46	0.74	-0.32

expressed as percentages, normalized to KYNA, rather than as absolute P_{app} values (Fig. 3). Analysis of both absolute and relative values revealed that they were higher than those of KYNA for all compounds. In the first approximation, it is notable that, despite the basic character of derivatives, they may exhibit measurable penetration across BBB-forming cerebral endothelial cells, even though, at pH 7.4, their protonated forms predominate. The substitution of the quinoline ring of **Compound 1** with a morpholinomethyl group (**Compound 2**) led to a pronounced increase *in vitro* BBB permeability, such as 131% and 199%. Replacement of the *N,N*-dimethylethyl chain in **Compound 2** with an *N*-ethylpyrrolidine moiety (**Compound 3**) would be expected to increase lipophilicity. However, the lipophilicity-enhancing effect of the additional two carbon atoms is presumably compensated by the slightly more basic character of the *N*-ethylpyrrolidine chain (pK_b 3.57) compared with the *N,N*-dimethylethyl chain (pK_b 4.01) (Williams, 2022). Consequently, no significant difference was observed between the logD values of the two compounds. Investigation of the effect of chlorine substitution at the 5, 6, 7, and 8 positions of the quinoline ring on passing through BBB-forming cerebral endothelial cells indicated that increasing the distance between the halogen from the quinoline nitrogen facilitates their *in vitro* BBB penetration, such as **Compound 4c** (142%), **4d** (211%), **4b** (257%) and **4a** (323%). The approximately two-

to three-fold increases *in vitro* permeability observed for **Compounds 4a, 4b, and 4d** relative to KYNA indicate a substantial improvement in BBB transport within the present experimental model and support the value of these structural modifications for further compound optimisation. However, the pharmaceutical significance of these differences should be interpreted cautiously. A proportional increase in P_{app} does not necessarily translate into a corresponding increase *in vivo* brain exposure or pharmacological effect, as these outcomes are additionally determined by systemic pharmacokinetics, protein and tissue binding, metabolism, clearance, and transporter-mediated processes. Therefore, the observed permeability improvements should primarily be considered useful for comparative ranking and prioritisation within the present compound series, while their impact on brain exposure and therapeutic potential requires confirmation in subsequent *in vivo* studies. Halogen substituents on the quinoline ring enhanced the penetration of given compounds across the cerebral endothelial cell layer compared to their unsubstituted parent, **Compound 2**, consistent with a previous study (Engkvist et al., 2003). The position of the chlorine substituent can significantly enhance (**4a, 4b, and 4d**) or only negligibly affect (**4c**) the penetration properties of the compounds relative to **Compound 2**. Analysis of the relationship between normalized P_{app} and the measured logD values of the studied compounds indicated a correlation

Fig. 3. Normalised *in vitro* permeability results of KYNA derivatives.

((Spearman's $r = 0.76$ which improved after (Spearman's $r_s = 0.89$) exclusion of **Compounds 4a** (see **Figure S21**). The relationship between relative permeability and predicted logBB was also analysed. A Spearman correlation coefficient of 0.81 indicated a moderate relationship between these two parameters (**Figure S22**) when **Compounds 4c** was excluded. The predictive logBB model, which accounts for the passive permeability of compounds through the BBB in the form of the distribution coefficient of the substance, considers ionisation only indirectly through logP and PSA, and it does not explicitly consider pKa-dependent ionisation at physiological pH, which may contribute to discrepancies between predicted and experimental BBB penetration. The resulting lower prediction accuracy is evident in the discrepancy between the calculated and measured orders of penetration capability for chlorine-substituted derivatives (**Table 4**, **Figure S21**), underscoring the importance of experimental confirmation of BBB permeability. It is important to note that additional factors potentially contributing to BBB penetration include polar-nonpolar surface area, steric hindrance, solubility, biochemical stability, active transport mechanisms, and the pKa–pKb values of protonatable and/or deprotonatable functional groups, which may influence the ionisation behaviour of the compounds (Engkvist et al., 2003; Singh and Kristam, 2020). Therefore, the structural and protonation states of the individual compounds at pH 7.4, including the locations of the charges, were determined using theoretical calculations by the Epik program. The relative abundances of the charge states assigned to the obtained enol-oxo tautomeric forms are summarised in **Table 5**, and the related structures are illustrated in **Table S3**. The most significant deviation was observed for **KYNA**, in which the negatively charged oxo tautomer is the most stable form (91.7%), which accounts for its low BBB permeability. In the case of **Compound 1**, in contrast to the other investigated compounds, the enol form is the most stable state (50.9%), bearing a single positive charge localized on the nitrogen atom of the *N,N*-dimethylamino group, which constitutes the most basic moiety among the derivatives containing this aminoalkyl carboxamide chain. In general, it can be concluded that the oxo tautomer represents the most stable state, with a single positive charge for the other derivatives. Interestingly, in the case of the **4a** isomer, the relative abundance of this form is (36.4%) comparable to that of its enol counterpart in the corresponding zwitterionic state (33.3%), in which the additional negative charge is located on the phenolate ion. Actually, **Compound 4a** had a significantly higher relative abundance of the zwitterionic enol tautomer than the other investigated derivatives, for which the abundance of this tautomeric/protonation state ranged from 3% to 13%. Although the higher permeability of **Compound 4a** coincided with a greater population of the zwitterionic enol tautomer, the present data establish an association rather than a causal relationship. The potential contribution of this tautomeric distribution to BBB permeability should therefore be regarded as a mechanistic hypothesis that requires further experimental validation. Based on the most stable chemical structures and protonation states at pH 7.4 obtained by computational calculations, the related polar and nonpolar/polar surface areas were determined, along with their relationship to the experimental normalized P_{app} values. The dominant tautomeric forms in proper protonation states of studied compounds were predicted to yield the largest PSA for **KYNA** and the

smallest for the **4a** isomer, and an exponential correlations were observed between the normalized P_{app} values and both PSA and NPSA/PSA (**Fig. 4**). The observed correlation underscores the importance of accurately determining the total solvent-accessible surface area, particularly for enhancing the BBB permeability of further **KYNA** derivatives, with due consideration of their most stable tautomeric forms and protonation states.

From a translational perspective, the present workflow may support early-stage lead optimisation by enabling **KYNA** derivatives to be prioritised on the basis of experimentally determined BBB permeability together with physicochemical and computational descriptors. Improved *in vitro* BBB permeability can serve as one criterion for identifying compounds with potentially more favourable brain exposure. In this context, the higher P_{app} values observed for **Compounds 4a**, **4b**, and **4d** relative to **KYNA** provide a rationale for their further preclinical evaluation, with **Compound 4a** warranting particular attention on the basis of its favourable *in vitro* BBB permeability within the present series. However, enhanced *in vitro* BBB permeability alone does not establish increased *in vivo* brain exposure, pharmacological efficacy, or therapeutic benefit, as these outcomes are additionally influenced by systemic pharmacokinetics, metabolic stability, plasma and brain tissue binding, and target-related pharmacodynamics. Moreover, transporter-mediated processes were not specifically characterised in the present study, which represents an additional limitation when interpreting the BBB permeability data. Consequently, the current permeability data do not allow the contribution of individual uptake or efflux transporters, including P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), to be distinguished from other determinants of BBB transport. Future studies incorporating transporter-specific assays or computational assessment of transporter liability would therefore be valuable for determining whether the investigated **KYNA** derivatives are subject to active uptake or efflux and for refining their prioritisation for further preclinical evaluation. Accordingly, the present platform should be regarded as an early-stage prioritisation tool that can reduce the number of candidates advanced to more resource-intensive pharmacokinetic, pharmacodynamic, and *in vivo* studies rather than as a substitute for such investigations. The applicability of the present workflow should also be interpreted within the chemical domain investigated in this study. The current compound set comprises a relatively small series of structurally related **KYNA** derivatives; therefore, the observed structure–property–permeability relationships cannot be assumed to be directly transferable to chemically diverse CNS-active compounds. Validation using larger and structurally more diverse compound sets will be required to establish the broader applicability and generalizability of the proposed workflow. In future studies, the experimentally determined permeability and physicochemical parameters generated by this workflow could also be integrated with pharmacokinetic and physiologically based pharmacokinetic (PBPK), including CNS-PBPK, models. Such integration could support the quantitative translation of *in vitro* permeability data toward predictions of systemic and brain exposure when combined with additional compound-specific parameters, including plasma and brain tissue binding, metabolic stability, clearance, and transporter-related data. Thus, the present experimental–computational workflow may provide input data for

Table 5

Predicted relative abundance (%) of the most stable tautomeric forms of studied compounds at different charge states.

Tautomer	Charge state	Relative abundance (%)							
		KYNA	1	2	3	4a	4b	4c	4d
Oxo	−1	91.7							
Enol	−1	7.8							
Oxo	+1		28.4	83.3	85.2	36.4	72.5	70.2	76.6
Enol	−1, +1		13.0	3.0	8.8	33.3	10.6	16.3	8.1
Oxo	0			10.5	2.9	5.2	9.6	8.6	9.9
Enol	+1		50.9						

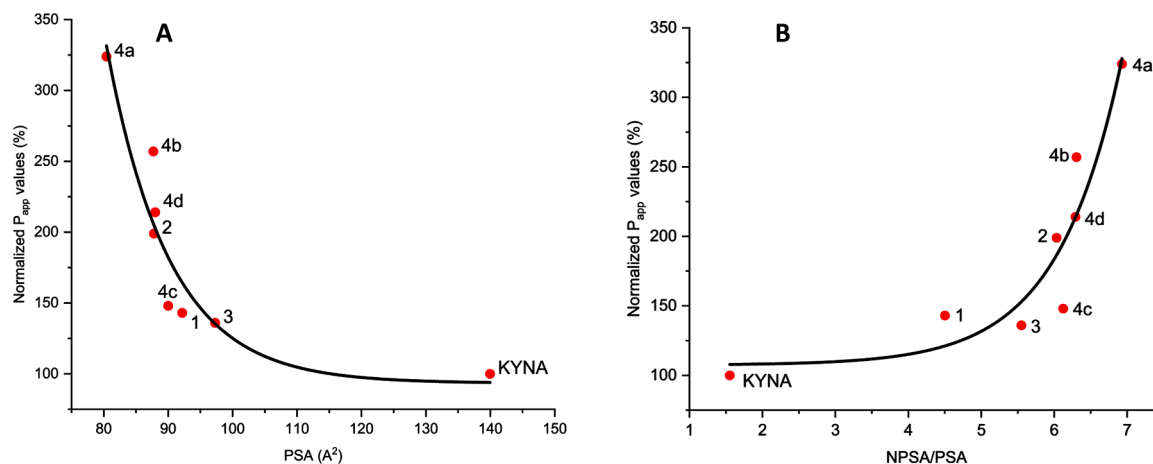


Fig. 4. (A) Correlation between the normalised P_{app} values (%) and PSA ($\text{\AA}^2$) ($R^2=0.88$), and (B) correlation between the normalised P_{app} values (%) and NPSA/PSA ($R^2=0.87$).

subsequent model-informed candidate selection, although the development and validation of such PK/PBPK models were beyond the scope of the present study.

4. Conclusion

This study establishes an integrated experimental–computational framework for the rational evaluation and optimisation of blood–brain barrier (BBB) permeability of kynurenic acid (KYNA) derivatives. By combining validated UHPLC–MS/MS quantification, experimental determination of lipophilicity using a modified shake-flask approach, *in vitro* BBB permeability measurements, and computational analysis of protonation states, tautomeric equilibria, and solvent-accessible surface areas, the proposed workflow provides a comprehensive link between molecular structure, physicochemical properties, and BBB permeability.

Targeted structural modifications markedly improved both lipophilicity and BBB permeability. Among the investigated compounds, the monochlorinated derivatives exhibited the greatest enhancement, with **Compound 4a** showing the highest permeability. Computational analysis further showed that its superior BBB transport was associated with a substantially higher population of the zwitterionic enol tautomer at physiological pH, suggesting a possible contribution of tautomeric equilibria to the observed permeability differences.

The observed relationships between experimental permeability, experimentally determined logD, predicted logBB, and solvent-accessible surface area descriptors demonstrate the value of integrating complementary experimental and computational approaches during lead optimisation. Overall, the integrated workflow provides a complementary approach for investigating structure–property–permeability relationships and supporting compound prioritisation within the present KYNA derivative series. Further validation using larger and structurally more diverse compound sets will be necessary to determine its applicability to broader CNS drug discovery contexts.

CRediT authorship contribution statement

Zahraa Ali: Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Bálint Lőrinczi:** Writing – original draft, Methodology. **Péter Simon:** Methodology. **Csilla Fazakas:** Methodology. **Imola Wilhelm:** Writing – original draft. **Ferenc Bogár:** Writing – original draft, Software, Formal analysis. **Linda Szilasi:** Formal analysis, Data curation. **István Ilisz:** Writing – review & editing. **László Vécsei:** Writing – review & editing, Funding acquisition. **István Szatmári:** Writing – review & editing, Funding acquisition, Conceptualization. **Róbert Berkecz:** Writing – review & editing, Writing

– original draft, Validation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ejps.2026.107674](https://doi.org/10.1016/j.ejps.2026.107674).

Data availability

Data will be made available on request.

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