Fluorosulfate-containing pyrazole heterocycles as selective BuChE inhibitors: structure-activity relationship and biological evaluation for the treatment of Alzheimer’s disease

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ABSTRACT

Novel scaffolds are expected to treat Alzheimer’s disease, pyrazole-5-fluorosulfates were found as selective BuChE inhibitors. Compounds K1–K26 were assayed for ChE inhibitory activity, amongst them, compound K3 showed potent BuChE inhibition (IC₅₀ = 0.79 μM and 6.59 μM). SAR analysis showed that 1-, 3-, 4-substituent and 5-fluorosulfate of pyrazole ring affected BuChE inhibitory activity. Molecular docking showed that the fluorosulfate increased the binding affinity of hBuChE through π-sulphur interaction. Compound K3 was a reversible, mixed and non-competitive BuChE inhibitor (Kᵢ = 0.77 μM) and showed remarkable neuroprotection, safe toxicological profile and BBB penetration. In vivo behavioural study showed that K3 treatment improved the Aβ₁₋₄₂-induced cognitive impairment, and significantly prevented the effects of Aβ₁₋₄₂ toxicity. Therefore, selective BuChE inhibitor K3 has potential to be further developed as AD therapeutics.

INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder, which causes severe impairment of cognitive functionality, neurodegeneration, and even Parkinsonian symptoms, leading the patient to a complete dependence even for accomplishing daily tasks. AD affects 50–60% of people with dementia, which the number of patients will increase from 55 million to an astonishing 151 million by 2050. Clinical evidences demonstrate that AD is a multicausal disease, and the patient’s age and gender are the main risk factors. The cholinergic hypothesis proposes that the degeneration of cholinergic neurons and the associated loss of cholinergic neurotransmission in the cerebral cortex are responsible for the deterioration of cognitive function observed in the brain of AD patients. Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are the two types of cholinesterases (ChEs) for hydrolysis of ACh in brain. AChE is mainly derived from regions of the neural synaptic junction and adult cerebral cortex that express intense activity, while BuChE is mainly derived from glial cells of the brain, maintaining a close spatial relationship of BuChE in glial cells and facilitating BuChE-mediated hydrolysis, thereby regulating local ACh levels, which in turn maintain normal cholinergic function.

However, the compensatory character of BuChE is greatly noticeable under pathological conditions. In the AChE-knockout mouse model, BuChE was proven to compensate for hydrolysing ACh due to the lack of AChE, thereby maintaining normal cholinergic function. The growing evidence has indicated that, with disease progression, the activity of AChE decreases to 10–15% of normal values in certain brain regions, while BuChE as a compensating enzyme is maintained at the normal or even higher level.

So far, only four ChE inhibitors (rivastigmine, galantamine, donepezil, tacrine) have been approved by FDA for the treatment of AD in clinic. However, they can only provide temporary and incomplete symptomatic relief. In addition, studies have shown that AChE can cause amyloid plaques, and the expression of BuChE is related to Aβ plaques, NFT and cerebral amyloid angiopathy. AChE and BuChE are still the most valuable and predominating targets for the discovery of new anti-AD agents. However, selective BuChE inhibitors are mainly pseudodirreversible car bamates and tacrine- or donepezil-based hybrids, but it is limited to discovering new drugs by simply structural modification. Few BuChE-targeted scaffolds were discovered, therefore, search for novel scaffolds with BuChE inhibition was requisite to the treatment of AD.

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structurally versatile scaffolds in chemical biology and drug development, including sultone scaffolds as BuChE inhibitors, sulfamides as novel pesticides \(^{24-29}\). Sulphonyl fluorides and fluorosulfates also exhibited excellent biological and pharmacological activities, such as BuChE inhibitors, potent antibacterial agents, cysteine protease SpeB inhibitors, human neutrophil elastase inhibitors, et al. \(^{31-34}\). Recently, a series of pyrazolyl fluorosulfates were synthesised through \(\text{SO}_2\text{F}_2\) mediated transformation of pyrazolones \(^{35-39}\). The primary screening showed that the 5-fluorosulfate of pyrazole had selective BuChE inhibitory activity compared to the pyrazole, and molecular docking showed that the fluorosulfate of precursor pyrazolone could increase the binding affinity of \(\text{hBuChE}\) through \(\pi\)-sulphur interaction between the sulphur and Trp82 (Figures 1 and S1). In this work, a series of pyrazole-5-sulfosulfates were synthesised for their ChE inhibitory activity, analysed for their structure-activity relationship (SAR), and evaluated the preliminary mechanism for treating AD.

**Materials and methods**

**Chemistry**

Recently, we constructed a class of novel \(N\)-heterocyclic molecules containing both pyrazole and fluorosulfate as versatile building blocks in the Suzuki coupling reaction and SuFEx click chemistry. Synthetic procedures and characterisation of compounds \(K1-K26\) were reported in the literature \(^{35}\). The purity (relative content) of active compounds was determined by HPLC on an Agilent 1200 instrument (column: Elite, RP-C18, 5 \(\mu\)m, 4.6 × 150 mm) through area normalisation method. TLC was carried out on pre-coated silica gel \(F_{254}\) glass plates with petroleum ether/ethyl acetate (10: 1) \(^{34}\). The same test conditions as indicated in the assay methodology. For the assessment of reversible and irreversible inhibitor binding at enzyme, different concentrations of compound \(K3\) were utilised.

**Kinetic studies of eqBuChE inhibition**

The same test settings were used for kinetic studies, with six concentrations of substrate (0.1–1 \(\mu\)M) and four concentrations of inhibitor (0–1.6 \(\mu\)M). The effect of different doses of compound \(K3\) on BuChE catalytic activity at 37°C was also investigated. The final concentration of enzyme was changed (0–0.18 \(\mu\)M/mL) under the same test conditions as indicated in the assay methodology. For the assessment of reversible and irreversible inhibitor binding at enzyme, different concentrations of compound \(K3\) (0, 0.4, 0.8, and 1.6 \(\mu\)M) were utilised.

**Molecular docking study**

The binding mechanisms of active compound to BuChE enzyme active sites were discovered using a structure-based in silico method. The Discovery Studio Client v18.1.0 (DS) CDOCKER was used to explain the SAR of series compounds and to guide the development of more effective and tangible BuChE inhibitors. As a template, the ligand binding to the crystal structure of \(\text{hBuChE}\) (PDB ID: 6QAA) was used. To assure the target enzyme’s integrity, it was produced using DS’s Prepare Protein. Full Minimisation of the Small Molecular in DS was used to prepare the ligand. Then, using CDOCKER, the title compounds were docked into the active site of the protein. The view results of molecular docking were extracted after the program running end, each docking result was analysed for interaction and their different pose. The most stable poses were those with the lowest -CDOCKER_INTERACTION_ENERGY values and
Cytotoxicity assays

Human hepatoblastoma cells HepG2 and human normal liver cells LO2 were cultured at 37 °C in DMEM containing 10% foetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified incubator with 5% CO2. The methyl thiazolyl tetrazolium (MTT) assay was used to assess cell cytotoxicity. In a 96-well plate, HepG2 cells and LO2 cells were injected at 1 × 10⁴ cells per well. The cells were treated with various substances that were diluted in DMEM for 24 h after being cultivated for 24 h. The cells were then treated for 4 h with 20 μL 0.5 mg/mL MTT reagent. The cell culture was withdrawn after 4 h, and 150 μL DMSO was added to dissolve the formazan. At 492 nm, the optical density was observed (OD492). Three independent tests were used to calculate cell viability.

Cell viability (%) = compound (OD492)/blank (OD492) × 100%.

Blank: cultured with fresh medium only.
Compound: treated with compounds or donepezil.

Neuroprotection assay

PC12 cells were distributed at a density of 1 × 10⁴ cells per well into 96-well microtiter plates. At time zero, cells were treated with a range of compounds K3 concentrations (1–25 μM) and kept for 3 h after being incubated overnight. The media were then changed with new media containing the medication as well as a cytotoxic stimulus in the form of 100 μM H2O2, which was left for another 24 h. The MTT assay was used to determine cell viability after 24 h. In the cells, 20 μL of 0.5 mg/mL MTT reagent was applied and incubated for 4 h. The cell culture was removed after 4 h, and 150 μL DMSO was added to dissolve the formazan. The optical density was measured at 492 nm (OD492). Three independent tests were used to calculate cell viability.

In vivo acute toxicity evaluation

A total of 20 mice weighing 20–25 g (F: M = 1: 1) were randomly allocated into two groups: control (n = 10) and experimental (n = 10). In a mixture of DMSO, PEG 400, and physiological saline (10/50/40, v/v/v), compound K3 was suspended. On the first day, the mice were intragastrically given the vehicle or test substance K3 0.45 g/kg after fasting for 8–12 h. For two weeks, the mouse’s behaviour, appearance, and body weight changes were examined and documented. GraphPadPrism8.0 software was used to compare and summarise the body weights of the mice in the control and experimental groups.

In vivo hepatotoxicity evaluation

Compound K3 was suspended in a mixed solution of DMSO, PEG 400, and normal saline (10/50/40, v/v/v) to assess in vivo hepatotoxicity in male ICR mice (20–25 g), which were also divided into blank and experimental groups. Mice were fasted for 24 h. Intragastrically, the combination was given at a dose of 30 mg/kg body weight with the same quantity of vehicle (po). With comparable kits, the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), both markers of liver damage, were assessed (EF551 and EF550 for ALT, EH027 and EH548 for AST). A biochemical analyser was used to process the data (Hitachi 7020, Japan). Mice were slaughtered and livers were obtained for immunohistochemical morphological evaluation after the last sample of postglobular blood. We isolated two 3-mm sections of each liver from the hilum to the edge of the left lateral lobe using an ultra-thin semi-automatic microtome (LeicaRM2245, Germany), immediately placed them in 10% buffered formaldehyde, fixed them for 2 days, and embedded them in paraffin blocks using a paraffin-embedding station (LeicaEG1150H, Germany). Subsequently, 5 μM sections were prepared from these paraffin sections, deparaffinized, stained with haematoxylin and eosin (HE) or using the periodic acid-Schiff glycogen staining method.

Animal studies

The National Institutes of Health Guide for the Care and Use of Laboratory Animals was followed for all experiments. The Animal Care and Use Committee of Anhui Medical University adopted the Measures for the Care and Treatment of Laboratory Animals. In Anhui Medical University’s animal centre, male ICR mice were employed (Hefei). Male mice (18–24 g) aged 6–8 weeks, 10 mice per cage, room temperature 22 ± 2 °C, light/dark (12:12 h) cycle. Prior to testing, these animals had access to food and water. Throughout all of the experiments, the room’s ambient conditions were maintained. The experiment period was 2 weeks. In at least three independent experiments, each animal was used as an individual sample. Each sample was set as three biological replicates, and the results of each experiment were averaged. When all the results were consistent, the results were considered credible.
temperature and relative humidity (50%) remained constant. Behavioural tests: MWM was used to test cognitive function, and mice were chosen at random for behavioural tests. 8–10 animals were used in each study group. The experimental time was from 08:00 to 14:00, and the mice were sacrificed by cervical dislocation shortly after the experiment ended. Positive and test chemicals were suspended in a combination of DMSO and 0.5% sodium carboxymethylcellulose (1/99, V/V) before the experiment for the Aβ1–42 oligomerization damage test, and 40 male mice were randomly allocated into five groups: (i) blank control group (po); (ii) saline (icv) + vehicle (proper amount, po); sham-operation group; (iii) oligomerized Aβ1–42 peptide (10 μg/mouse, icv) + vehicle (proper amount, po); model group; (iv) oligomerized Aβ1–42 peptide (10 μg/mouse, icv) + donepezil (15 mg/kg, po), donepezil group, (v) oligomerized Aβ1–42 peptide (10 μg/mouse, icv) + donepezil (15 mg/kg, po), K3 group. Aβ1–42 aggregation was induced by dissolving the Aβ1–42 peptide in DMSO as a stock solution of 5 mM and incubating it in saline at a final concentration of 2 mg/mL for 24 h at 37 °C. The mice participated in a water maze experimental behavioural investigation on days 10–15, which included a 5-day learning and memory training and a test evaluation on day 6. MWM is made up of a water-filled pool (gray, circular, 1.20 m in diameter, 0.60 m in height) and a platform that can be adjusted in height and moved. Using a video tracking system, the pool was divided into four equal quadrants (compass position: NE, NW, SE, SW) (SMART, version 3.0; Panlab, Spain). To prevent animals from jumping out, the pool was filled with water up to 48 cm below the edge, and the water temperature was kept at 22 ± 1 °C. The escape platform was made of transparent plexiglass (11 cm in diameter and 47 cm in height) and was soaked 1 cm below the water level in a fixed position (the middle of the northwest quadrant, that is, the target quadrant). The pool is in a larger area with no light shadow on the pool water surface and four reference objects on the pool wall with distinct geometric designs. During the training, the mice were randomly placed in the water facing the pool wall from one of four quadrants (NE, NW, SE, SW), with the platform in the southeast quadrant, and each experimental mouse swam for a total of 60 s to find the hidden platform. If the mouse was unable to locate the platform in the pool or climb it within 60 s of entering the water, the mouse was instructed to stand on the platform for 15 s. The average speed, the time to reach the hidden platform (i.e., escape latency), the distance to reach the hidden platform, and the distance in the target (NW) area were all recorded. The platform was removed from the pool on day 6 (24 h following the last training session) and a probing trial (Drogoff test) was conducted. Each mouse was allowed to swim once, and if the previous platform position was not found within 60 s, a latency score of 60 s was given to measure the latency to first cross the previous platform position (i.e., the target area), the number of times it crossed the target area, the time spent in the target NW quadrant, the total distance, the distance spent in the NW quadrant, the entry into the NW quadrant, and the mean speed, and compared across experimental groups.

In the Aβ1–42 oligomeric damage experiment, all mice were sacrificed after the behavioural research was completed, and the brains were collected to assess the total content of Aβ1–42 using a mouse ELISA kit. Each brain tissue sample was thoroughly homogenized in a grinder with 10 times PBS (pH = 7.4 ± 0.1) before being centrifuged for 5 min at 5000 × g. The supernatant was separated for use. The detection technique followed the directions exactly, and the standard curve was created. Brain tissue Aβ1–42 content was calculated according to the linear regression equation. All values were expressed as mean ± SEM using GraphPad Prism 8.0 software.

Statistical analysis
Data are reported as mean ± SEM of at least three independent experiments and data analysis was performed with GraphPad Prism 8 software.

Results and discussion

Chemistry

Recently, a class of novel N-heterocyclic molecules containing both pyrazole and fluorosulfate was constructed as versatile building blocks in the Suzuki coupling reaction and SuFEx click chemistry.10 The novel fluorosulfate-containing pyrazole heterocycles (compounds K1–K26 as shown in Scheme 1) were synthesised through the reaction of pyrazolones with SO2F2 under 1.5 equivalents of DIPEA in CH2Cl2 in good to excellent yields.

Inhibitory activity against AChE and BuChE

The materials M1–M4 and pyrazole-5-fluorosulfates K1–K26 were evaluated for their activity with Electrophorus electricus AChE (EeAChE) and equine BuChE (eqBuChE), using modified Ellman’s method. The IC50 values were obtained and compared to the reference rivastigmine, and donepezil, which is a selective AChE inhibitor that simultaneously binds to catalytic active and peripheral anionic sites (PAAS). Enzymatic assays revealed that all pyrazole-5-fluorosulfates K1–K25 showed inhibitory activities against AChE and BuChE except for compound K19, while their precursors 1,3-disubstituted-1H-pyrazol-5(4H)-ones M1–M4 showed weak inhibitory activity against AChE and BuChE (IC50 > 20 μM). Compound K22 showed selective AChE inhibition (IC50 values for AChE and BuChE, 2.60 and 17.99 μM, respectively), while the other exhibited moderate to strong inhibitory activity against showing selectivity towards BuChE. It was obvious from the data that compound K3 showed the best inhibitory activity against BuChE with IC50 value of 0.79 μM. Inspection of the chemical structures, it can be concluded that the BuChE inhibitory activity was affected by the substituent groups at the 1-, 3- or 4-positions of pyrazole fluorosulfates (Table 1). From Table 1, it is intuitive that the substituent of 1-aryl ring and 3-position at the pyrazole ring plays important role on the activity.

SARs of pyrazole-5-fluorosulfates for BuChE inhibition

As shown in Scheme 1 and Table 1, pyrazole-5-fluorosulfate derivatives exhibited selective BuChE inhibitory activity except for compound K19. The structure-activity relationship (SAR) was further analysed. Firstly, the substituent at 1-phenyl ring of pyrazole-5-fluorosulfate affects the BuChE inhibitory activity: (i) the order of substituent at 4-position: -Cl > -F > -Br > -H > -CH3, such as K3 (-Cl, 0.79 μM) > K2 (-F, 1.73 μM) > K1 (-H, 2.28 μM) > K4 (-CH3, 2.35 μM) for 3-phenyl, K15 (-Cl, 5.26 μM) > K14 (-F, 5.50 μM) > K16 (-Br, 7.64 μM) > K13 (-H, 9.16 μM) for 3-methyl, K21 (-F, 6.90 μM) > K20 (-H, 11.25 μM) > K22 (-CH3, 17.99 μM) for 3-isopropyl; (ii) 3,4-diMe > mono-Me: K5 (-diMe, 2.23 μM) > K4 (4-Me, 2.35 μM) for 3-phenyl, K18 (–diMe, 3.31 μM) > K17 (3-Me, 19.18 μM) for 3-methyl, K23 (–diMe, 3.98 μM) > K22 (4-Me, 17.99 μM) for 3-isopropyl; (iii) when 1-phenyl was substituted by

Statistical analysis

Data are reported as mean ± SEM of at least three independent experiments and data analysis was performed with GraphPad Prism 8 software.
smaller group (t-Bu), BuChE inhibition decreased, such as K1 > K9, K13 > K19.

Secondly, the effect of substituent at 3-position of pyrazole on BuChE inhibition was observed: (i) –phenyl > –CH3 > –i-Pr, such as K1 (2.28 µM) > K13 (9.16 µM) > K20 (11.25 µM) for 1-phenyl, K2 (1.73 µM) > K14 (5.50 µM) > K21 (6.90 µM) for 4-F-phen-1-yl, K3 (0.79 µM) > K15 (5.26 µM) for 4-Cl-phen-1-yl, K4 (2.35 µM) > K22 (17.99 µM) for 1–4-Me-phenyl, K5 (2.23 µM) > K18 (3.31 µM) > K23 (3.98 µM) for 1–3,4-diMe-phenyl, K9 (30.9%) > K19 (no activity) for 1-t-butyl; (ii) when the substituent at 3-position was n-propyl, compounds exhibited potent BuChE inhibitory activity (IC50 = 4.59 and 3.47 µM for K24 and K25, respectively); (iii) cyclisation of the i-propyl (IC50 = 11.25 µM for K20) led to decrease the activity (IC50 = 14.36 µM for K26).

Finally, the multi-substituent of pyrazole ring led to decrease BuChE inhibitory activity, for example, inhibitory rates of compounds K11 and K12 were 45.2% and 20.7%, respectively. All in all, the SAR of pyrazole-5-sulfofluoridate scaffold is illustrated in Figure 2.

Inhibition of hBuChE and hAChE
In order to determine the potency and selectivity of compounds K3 for the human enzymes, ChE inhibitory activity was detected on hAChE and hBuChE. As shown in Table 2, Compared to the positive control rivastigmine, compound K3 showed stronger inhibitory effect on hBuChE (IC50 = 6.59 µM) and have inhibitory effect on hAChE (31.65% inhibitory rate at 20 µM). Hence, compound K3 was found as a selective hBuChE inhibitor.

Kinetic study of eqBuChE inhibition
Potent inhibitor K3 was subjected to enzyme kinetics analysis to determine the kinetics of BuChE inhibition43–45. As shown in
Table 1. Inhibitory activities against eeAChE and eqBuChE of compounds M1–M4 and K1–K25 (IC\textsubscript{50}, µM or % inhibition at 20µM).\textsuperscript{a}

| Compound | AChE\textsuperscript{b} | BuChE\textsuperscript{c} |
|----------|-----------------|-----------------|
| M1       | 8.8 ± 2.6%      | na              |
| M2       | 37.0 ± 3.3%     | 27.7 ± 11.7%    |
| M3       | 26.9 ± 1.0%     | 25.4 ± 5.4%     |
| M4       | 10.7 ± 1.9%     | 44.1 ± 13.8%    |
| K1       | 33.6 ± 1.6%     | 2.28 ± 0.64     |
| K2       | 27.5 ± 2.7%     | 1.73 ± 0.62     |
| K3       | 26.3 ± 1.4%     | 0.79 ± 0.32     |
| K4       | 25.9 ± 0.9%     | 2.35 ± 1.02     |
| K5       | 24.4 ± 2.1%     | 2.23 ± 0.31     |
| K6       | 25.7 ± 1.5%     | 4.79 ± 1.9%     |
| K7       | 19.39 ± 0.01    | 9.89 ± 0.87     |
| K8       | 25.1 ± 1.2%     | 5.84 ± 0.87     |
| K9       | 10.1 ± 0.4%     | 30.9 ± 1.5%     |
| K10      | 32.0 ± 1.2%     | 6.92 ± 1.41     |
| K11      | 7.2 ± 1.0%      | 45.2 ± 1.8%     |
| K12      | 42.8 ± 0.8%     | 20.7 ± 1.7%     |
| K13      | 37.5 ± 1.4%     | 9.16 ± 0.53     |
| K14      | 48.2 ± 1.0%     | 5.50 ± 0.09     |
| K15      | 42.5 ± 1.3%     | 5.26 ± 0.39     |
| K16      | 37.2 ± 0.7%     | 7.64 ± 0.41     |
| K17      | 33.4 ± 1.3%     | 10.1 ± 0.4%     |
| K18      | 8.1 ± 0.5%      | 3.31 ± 0.70     |
| K19      | na              | na              |
| K20      | 41.6 ± 0.7%     | 11.25 ± 0.11    |
| K21      | 7.79 ± 0.07     | 6.99 ± 0.42     |
| K22      | 26.0 ± 0.27     | 17.99 ± 0.34    |
| K23      | 21.4 ± 1.1%     | 3.98 ± 0.09     |
| K24      | 41.8 ± 0.2%     | 4.59 ± 0.06     |
| K25      | 10.3 ± 0.6%     | 3.47 ± 0.04     |
| K26      | 4.82 ± 0.07     | 14.36 ± 1.08    |
| Donepezil| 0.071 ± 0.02    | 9.66 ± 0.60     |
| Rivastigmine| 16.13 ± 1.21  | 0.063 ± 0.03  |

\textsuperscript{a}Each IC\textsubscript{50} value is the mean ± SEM from at least three independent experiments; \textsuperscript{b}AChE from electric eel; \textsuperscript{c}BuChE from horse serum; \textsuperscript{d}na, no inhibitory activity (%) against either eeAChE or eqBuChE at 20 µM.

Table 2. Inhibitory activity on hAChE and hBuChE.\textsuperscript{a}

| Compound | hAChE\textsuperscript{b} | hBuChE\textsuperscript{c} |
|----------|-----------------|-----------------|
| Donepezil| 0.008 ± 0.004   | 12.42 ± 0.90    |
| Rivastigmine| 10.45 ± 2.95%  | 7.77 ± 2.92    |
| K3       | 31.65 ± 5.74%   | 6.59 ± 3.54     |

\textsuperscript{a}Each IC\textsubscript{50} value is the mean ± SEM from at least three independent experiments; \textsuperscript{b}hAChE from recombinant human AChE (hAChE); \textsuperscript{c}hBuChE from human serum.

Molecular docking of compound K3

The fluorosulfate of pyrazolone was observed to increase the binding affinity with hBuChE in Figure S1, however, a re-docking protocol was carried out to understand the binding modes of K3 targeting hBuChE in detail. As shown in Figure 4(A,B), compound K3 could insert into the binding groove of hBuChE, forming multiple interactions via π–π interaction between the benzene ring and Gly116 and Trp231 and Phe329, halogen bond between the chlorine and Asn83. Moreover, the fluorosulfate (SO\textsubscript{2}F) moiety could form π-Sulphur interaction with Trp82 and carbon hydrogen bond interaction with His438, which is consistent with the result in Figure S1.

Cytotoxicity assays

The cytotoxicity of active compounds was tested using human normal hepatocyte L02 and human hepatoblastoma HepG2 by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. As shown in Figure 5(A,C), at the concentration of 25 µM, the cell viability of all compounds was greater than 80% for HepG2 and LO2 cells, indicating that compound K3 has a wide range of therapeutic safety. On the basis of enzyme inhibition activity and cytotoxicity, compound K3 was selected as the representative. As shown in Figure 5(B,D), the cell survival rate of compound K3 was maintained at 94.4% and 87.9% even at 50 µM. The results showed that compound K3 had broad therapeutic safety against L02 and HepG2 cells.

Neuroprotective study

The protective effects of compound K3 against free radicals damage were assessed by measuring the ability of the compound to protect against H\textsubscript{2}O\textsubscript{2} injury. After 100 µM H\textsubscript{2}O\textsubscript{2} exposure, cell viability as determined by MTT reduction was obviously decreased to 46.5% (p < 0.01 vs control), manifesting high sensitivity to H\textsubscript{2}O\textsubscript{2}-induced injury. Compound K3 showed protective effects in a dose-dependent manner against H\textsubscript{2}O\textsubscript{2}-induced PC12 cell injury (Figure 6). At a concentration of 25 µM, compound K3 showed neuroprotective effects and was slightly stronger than the positive control donepezil, with cell viability of 69.3%. When the concentration was reduced to 5 µM, the cell viability decreased to 55.9%. It results showed that compound K3 had a good protective effect on H2O2-induced PC12 cell damage.
**PAMPA-BBB penetration assay**

It is essential to evaluate the BBB penetration capacity of active compounds into the central nervous system as the therapeutic target of the anti-AD agent. PAMPA (parallel artificial membrane permeation assay) is an *in vitro* model of passive diffusion, which was used to predict BBB penetration. Hence, BBB penetrating ability of compound **K3** was estimated using PAMPA-BBB model. To validate the experimental procedure, we chose six commercial drugs with reported values. A plot of experimental versus bibliographic data presented a good linear correlation, $P_{\text{e}}(\text{exp.}) = 0.9499 P_{\text{e}}(\text{bibl.}) + 0.0982$ ($R^2 = 0.9745$) (Figure 7(B)). **K3** was tested following this procedure, and the results in Figure 7(A) showed that permeability values of tested compounds are above $4 \times 10^{-6}$ cm/s, indicating the potential BBB penetration of **K3**.

**In vivo acute toxicity evaluation**

The body weight of mice in the vehicle group and **K3** groups increased throughout the 14 days prior to treatment, as shown in Figure 8(A), although the differences in body weight changes were not significant, showing that compound **K3** was well tolerated at high dosages (1.0 g/kg). As shown in Figure 8(B,C), serum ALT and AST levels were directly proportional to the degree of liver injury. No significant difference was found ($p > 0.05$) between the vehicle group and the **K3** group at each time point, nor within the **K3** group at each time point. The hepatotoxicity of compound **K3** was detected morphologically using immunohistochemistry. Figure 8(D) displays the paraffin sections of the control group and the compound **K3** (30 mg/kg) after 36 h of administration. The findings demonstrated that compound **K3** had great safety in vivo since there was no core necrosis or evident steatosis in and around the intermediate zone around the hilum.

**Behavioural studies**

We established a model of cognitive impairment generated by Aβ1–42 (intracerebroventricular (icv) injection) to explore compound **K3**’s anti-AD efficacy. On day 1, amyloid peptide (10 μg per mouse) was injected into the ventricles of 30 mice,
while a sham group was established, with the same amount of saline put into the ventricles. From Day 3 to Day 14, donepezil (15 mg/kg, as positive groups) and K3 (10 mg/kg) were administered (po). Throughout the administration period, the animal’s health and weight were monitored on a daily basis (Figure 9(B)). Compound K3 had no effect on body weight increase, and there was no significant difference from the control group, indicating that K3 is quite safe. From Day 10 to Day 14, behavioural studies were conducted. The Morris water maze test (MWM) was used. MWM was a spatial learning test that used distal cues to navigate from the starting point around the open swimming field to the underwater escape platform. MWM was primarily employed to investigate the impact of reducing the time it took to reach the escape platform (also known as escape time latency) on long-term memory. MWM test included learning behaviour test on Days 10–14 and probe test on Day 15. Intraventricular injection of normal saline had no effect on the cognitive and learning abilities of mice, and there was no difference compared to the blank group in terms of the undifferentiated alternating behaviour, latency to reach the target, or confusion, as shown in Figure 9(C). The mice in the model group had much lower learning and memory abilities than those in the control group. As seen in Figure 9(D–F), the donepezil group was able to dramatically reduce the time it took to discover the platform and enhance the time spent on it when compared to the model group. Compared with the donepezil group, K3 shortened the latency, simplified the movement trajectory to the platform, improved the interaction ability, and the overall target quadrant preference (the number of crossing the platform and the swimming time in the target quadrant), indicating that both K3 (10 mg/kg) and donepezil (15 mg/kg) significantly improved the Aβ1–42-induced cognitive dysfunction.

The mice were slaughtered at the end of the behavioural trial, and Aβ1–42 levels were evaluated using a mouse Aβ1–42 ELISA kit. As shown in Figure 10(A,B), the total levels of Aβ1–42 peptides in the icv Aβ1–42 group were significantly higher than the control or sham groups, indicating that the modelling was successful, and the Aβ1–42 peptides in the mice treated with donepezil or K3 were significantly decreased (14.5% and 17.6%, respectively).
consistent with the results of behavioural experiments, indicating that compound \(K3\) can further exert a neuroprotective effect on \(A\beta_{1-42}\) toxicity by reducing BuChE levels, thereby effectively improving the cognitive function of AD mice.

**Conclusion**

A series of pyrazole-5-fluorosulfate derivatives were screened as selective BuChE inhibitors except for compound \(K19\), amongst them, compounds \(K3\) showed potent BuChE inhibitory activity (IC\(_{50} = 0.79\) \(\mu\)M). The structure-activity relationship (SAR) for pyrazole-5-fluorosulfates showed that the substituent at 1-phenyl ring affects the BuChE inhibitory activity: (i) 4-Cl > 4-F > 4-Br > 4-H > 4-CH\(_3\), such as \(K3 > K2 > K1 > K4\) for 3-phenyl, \(K15 > K14 > K16 > K13\) for 3-methyl, \(K21 > K20 > K22\) for 3-isopropyl; (ii) 3,4-diMe > mono-Me: \(K5 > K4\) for 3-phenyl, \(K18 > K17\) for 3-methyl, \(K23 > K22\) for 3-isopropyl; (iii) substituted t-Bu decreased BuChE inhibition, such as \(K1 > K9, K13 > K19\); the order of 3-substituted pyrazole: (iv) –phenyl > –CH\(_3\) > –i-Pr, such as \(K1 > K13 > K20\) for 1-phenyl, \(K2 > K14 > K21\) for 4-F-phen-1-yl, \(K3 > K15\) for 4-Cl-phen-1-yl, \(K4 > K22\) for 4-Me-phen-1-yl, \(K5 > K18 > K23\) for 3,4-diMe-phen-1-yl, \(K9 > K19\) for 1-t-butyl.

Molecular docking showed that compound \(K3\) could nicely insert into the binding groove of \(hBuChE\) (IC\(_{50} = 6.59\) \(\mu\)M for \(hBuChE\)), forming \(\pi-\pi\), \(\pi-S\) and halogen bond multiple interactions.
According to kinetic studies, compound K3 (Ki = 0.77 M) was a reversible, mixed, and non-competitive BuChE inhibitor. Additionally, compound K3 demonstrated exceptional neuroprotective efficacy and moderate BBB penetration potential. Compound K3 had good neurological and hepatic safety and was tolerated up to a dose of 1.0 g/kg, according to in vitro and in vivo safety experiments. In a following in vivo behavioural research, K3 therapy increased the Aβ1-42-induced cognitive impairment, greatly reduced Aβ1-42 toxicity, and nearly recovered cognitive function. Furthermore, the evaluation of the Aβ1-42 total amount confirmed its anti-amyloidogenic effects better than the positive donepezil. As a result, compound K3 has the potential to be further developed into an effective therapy for the treatment of AD.

The data of ChE inhibition assays that were used to calculate the IC50 and enzyme kinetics, the copies of representative 1H and
\(^{13}\)C NMR spectra and the copies of HPLC spectra can be found in Supplementary materials.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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**Figure 10.** The A\(_{1-42}\) total amount was quantified by using a mouse A\(_{1-42}\) ELISA kit. (A) Standard curve; (B) A\(_{1-42}\) total amount in mice brains of different groups. Calculate brain tissue A\(_{1-42}\) content according to linear regression equation, data are presented as mean ± SEM (\(n = 8\); \(* * p < 0.001\) (vs control group), \(\# \# p < 0.001\) vs \(\# p < 0.05\), \(\# \# \# p < 0.01\) vs A\(_{1-42}\) peptide model group).
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