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ORIGINAL ARTICLE

Synthesis and anticholinesterase activity of novel non-hepatotoxic naphthyridine-11-amine derivatives

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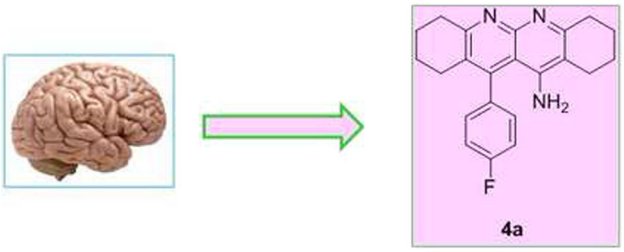
Abstract

In the present study, 14 novel naphthyridine-11-amine derivatives were synthesized and their inhibitory effects on acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) were evaluated. 12-(4-Fluorophenyl)-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1, 8]naphthyridin-11-amine (**4a**) was found to be the most potent AChE inhibitor with IC_{50} value of 0.091 μM, and 12-(2,3-dimethoxyphenyl)-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1,8]naphthyridin-11-amine (**4h**) exhibited the strongest inhibition against BuChE with IC_{50} value of 0.182 μM. Additionally, hepatocellular carcinoma (HepG2) cell cytotoxicity assay for the synthesized compounds was investigated and the results showed negligible cell death. Log *P* values of the synthesized compounds were also calculated using ChemSketch program. Moreover, the blood–brain barrier (BBB) permeability of the potent AChE inhibitor (**4a**) was assessed by the widely used parallel artificial membrane permeability assay (PAMPA-BBB). The results showed that **4a** is capable of crossing the BBB.

Graphical abstract



4a, IC_{50} = 0.091 μM AChE; IC_{50} = 0.773 μM BuChE
Non-hepatotoxic, $P_e = 5.42 \times 10^{-6}$ cm s^{-1}

Keywords Naphthyridine · Anticholinesterase activity · Cell cytotoxicity · Blood–brain barrier permeability

Introduction

Alzheimer's disease (AD) is a progressive nervous affecting disease with a high incidence in elderly people, leading to both physical and mental retardation and eventually death [1]. According to health organization reports, it is estimated that the number of people with the AD and other dementia and similar diseases is 47 million, and it is assumed that this number will triple by 2050 [2]. Pathologically, the AD is characterized by loss of cholinergic neurons, formation of hyperphosphorylated tau protein in intracellular neurofibrillary tangles (NFT) [3], abnormal processing of amyloid precursor protein (APP) and extracellular accumulation of β-amyloid protein [4, 5]. Today, one approach to explain at least several aspects of the pathology of the AD is covered by the cholinergic hypothesis [6]. The AD is characterized by the loss of cholinergic neurotransmitters, particularly

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acetylcholine, in both cortical and hippocampal regions. Cholinergic neurons play an important role in learning and memory by performing acetylcholine (ACh) synthesis [7]. In particular, the reduction in neurotransmitter acetylcholine (ACh) concentration is considered to be a fundamental factor for progressive cognitive impairment [7]. For this reason, the primary treatment strategy for the symptomatic treatment of AD is the use of cholinesterase inhibitors. The inhibition of these enzymes can prevent the reduction in the ACh level, which can compensate for the deficit in cholinergic neurons [8]. Acetylcholinesterase (AChE) is an enzyme of the class of cool hydrolases, a drug target for Alzheimer's disease [9]. AChE, which provides acetylcholine hydrolysed to choline and acetic acid, thus controls the level of ACh and regulates the acetylcholine effect. This enzyme is widely deployed along the body and is the most common cholinesterase in the human brain [10]. AChE is a membrane-bound enzyme, found mostly in cholinergic neurons in the body and also in the brain and muscles. Butyrylcholinesterase (BuChE) is expressed in the neuroglia, although it is not in the brain and cholinergic neurons, and is found in the liver, heart, intestine, serum, kidney and lung [11].

Tacrine is well known and used as the first synthetic cholinesterase inhibitor (ChEI), and other ones such as galantamine, donepezil and rivastigmine (Fig. 1) have been started to be used later for AD therapy [12]. The use of these drugs is restricted due to gastrointestinal problems and side effects such as hepatotoxicity [13]. For this purpose, many new ChEIs have been isolated from natural sources [14] or synthesized new active compounds [15]. Despite its ability to function as a very good inhibitor, the tacrine molecule cannot be used therapeutically due to hepatotoxicity. The adverse side effects of ChEIs present a major research area for researchers to synthesize novel non-toxic synthetic cholinesterase inhibitor drugs [16]. Tacrine, 9-amino-1,2,3,4-tetrahydroaminoacridine, which is licensed as the cholinesterase inhibitor by the US Food and Drug Administration for the treatment of Alzheimer's disease is the first drug [17]. The hepatotoxicity of the tacrine is due to the elevation of the serum alanine aminotransferase level [18], which causes the tacrine to be used in limited clinical practice. Consequently, the pharmaceutical market was withdrawn shortly after tacrine approval [19]. However, the tacrine with the high cholinesterase inhibitor feature has not been overlooked and has been used extensively and successfully in medical chemistry using it in hybrid [20] or multitarget compounds [21]. To combine tacrine's AChE inhibition with other pharmacological properties and to enhance its efficacy, tacrine is coupled to covalent bonds to other pharmacophores such as CB1 receptor antagonists and an M1 agonist [22, 23]. In this context, the development of tacrine analogues [24] is of great interest due to its inhibitory nature at low micromolar concentrations, mainly through the interaction of π-π stacking with Trp84 residues [25]. Various investigations have shown that tacrine-induced oxidative stress can be treated with radical scavengers such as hepatocytes, vitamin E or other dithioles. It is also known that some NO donors play a beneficial role in preventing hepatotoxicity. Tacrine hybrids containing radical scavengers, anti-oxidative properties and anti-amyloid aggregation can help prevent oxidative stress and fibril accumulation [26]. It is known to use different heterocyclic structures instead of the benzene ring of the tacrine to reduce toxicity and provide high anticholinesterase activity and selective peripheral attachment [27].

In this study, novel naphthyridine-11-amine, which is N-heterocyclic ring condensed tacrine, derivatives (**4a–k** and

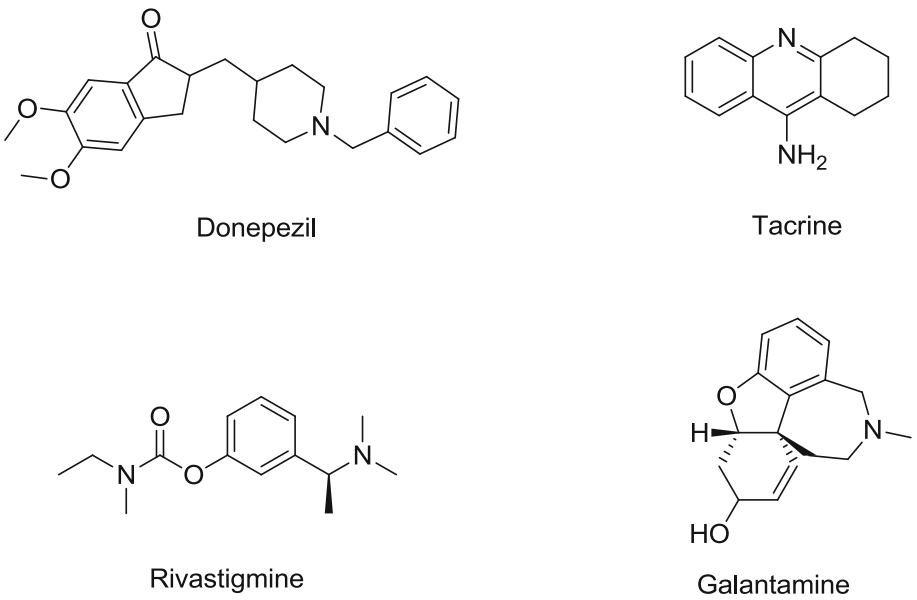
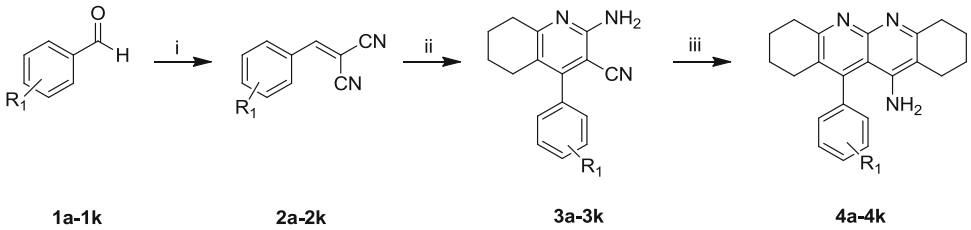
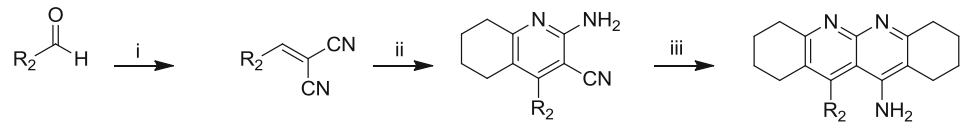


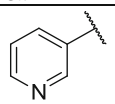
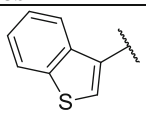
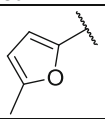
Fig. 1 Synthetic cholinesterase inhibitors



Scheme 1 Synthesis of new naphthyridine-11-amine derivatives. Reaction conditions: (i) malononitrile, EtOH, piperidine, 1 h, 80 °C; (ii) cyclohexanone, NH_4OAc, benzene, 10 h, 100 °C; (iii) cyclohexanone, $ZnCl_2$, 4 h, 140 °C

Compound	R_1	Compound	R_1
4a	4-F	**4g**	4-OCH_3
4b	3-Cl	**4h**	2,3-diOCH_3
4c	4-Cl	**4i**	4-CH_3
4d	3,4-diCl	**4j**	4-H
4e	3-Br	**4k**	4-NO_2
4f	4-Br		



Compound	8a	8b	8c
R_2			

8a–c) were synthesized and their anticholinesterase activities and hepatotoxicity were investigated. The octanol/water partition coefficient (log *P*), which plays an important role in the development of new drugs, has also been calculated. Moreover, the blood–brain barrier (BBB) permeability of the novel compounds was assessed by the widely used parallel artificial membrane permeability assay (PAMPA-BBB).

Result and discussion

Chemistry

The synthetic procedures are shown in Scheme 1. **2a–k** and **6a–c** were synthesized from aldehyde derivatives (**1a–k** and **5a–c**) and malononitrile [28]. The nitrile derivatives were reacted with cyclohexanone and ammonium acetate to get aminocyanopyridine derivatives (**3a–k** and **7a–c**) [29]. The aminocyanopyridines were reacted with cyclohexanone using $ZnCl_2$ as a catalyst to obtain the final products (**4a–k** and **8a–c**) [30].

All new compounds (except the compounds 4a, 4c and 4g, [31]) were characterized by spectroscopic methods such as ^{1}H NMR, ^{13}C NMR, IR, MS and elemental analysis. MS spectra of the synthesized compounds are given in the Supporting Information. In the infrared spectra of the synthesized compounds, the NH stretch of the NH_2 group present in the final products shows an absorbance between 3480 and 3280 cm^{-1}, while the CN moiety stretch of the intermediate product shows the absorbance between 2200 and 2220 cm^{-1}. Tacrine was synthesized by different groups in the $ZnCl_2$ catalyst as before. Yang et al. [31] observed both tacrine and highly rearranged product by using $ZnCl_2$. However, in the study of Mao et al. [30], tacrine molecule was obtained as a single product. As can be seen in Fig. 2, NH_2 group gave a singlet signal at 5 ppm at ^{1}H NMR spectrum of **3b**, whereas it shifted to 4 ppm at ^{1}H NMR spectrum of **4b**. In addition, the number of aliphatic protons showed an increase in ^{1}H NMR spectrum of **4b**. Consequently, our results are consistent with the structure proposed by Mao et al. From the ^{1}H NMR spectra, the signals of the NH_2 protons in the aminocyanopyridine derivatives were observed between 5.00 and 5.40 ppm, while the NH_2 protons of the naphthyridine-11-amine derivatives were observed between 4.00 and 4.20 ppm. The signals of aromatic and aliphatic hydrogens were observed between 6.10 and 8.90 ppm and 1.65 and 3.40, respectively. From the ^{13}C NMR spectra, the signals of aromatic carbons can

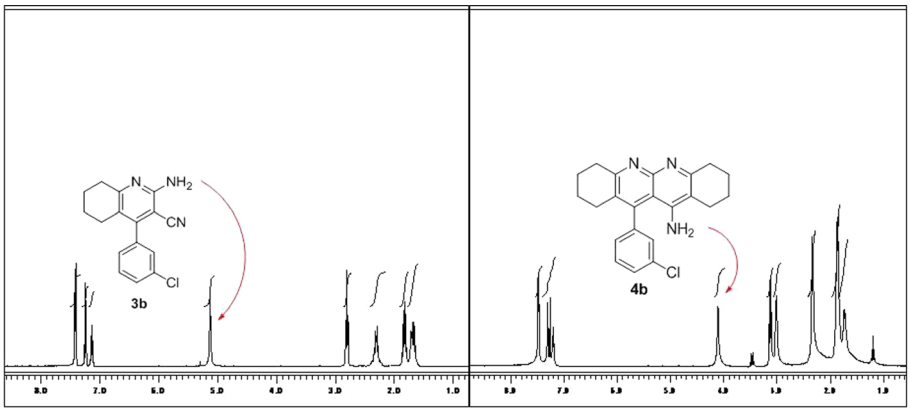


Fig. 2 Chemical shift of NH_2 group at the **3b** and **4b**

also be seen between 105 and 164 ppm. Signals of aliphatic carbons were also recorded between 15 and 55 ppm.

Biological activity

Cholinesterase inhibitory activity

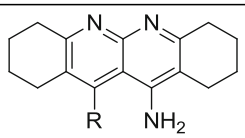
The inhibitory effects of compounds on the acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) were evaluated using Ellman's protocol [32]. Tacrine, galantamine and donepezil were used as reference compounds. As shown in Table 1, the synthesized compounds exhibited low micromolar inhibitory potencies against AChE and BuChE with the IC_{50} values ranging from 0.091 to 1.197 μM and from 0.182 to 4.881 μM, respectively. Among them, **4a** exhibited the strongest inhibition against AChE with an IC_{50} value of 0.091 μM, which is 11-fold more than that of galantamine (IC_{50} = 1.054 μM). Furthermore, **4a** has similar AChE inhibitory activity compared to that of donepezil (IC_{50} = 0.101 μM), which is well known as AChE inhibitor, and it has similar inhibition with standard molecule tacrine (IC_{50} = 0.055 μM). **4h** exhibited the strongest inhibition against BuChE with an IC_{50} value of 0.182 μM, which is 100-fold more than that of galantamine (IC_{50} = 18.13 μM) and 14-fold more than that of donepezil (IC_{50} = 2.680 μM), but it showed less activity with the tacrine (IC_{50} = 0.032 μM).

The following results of the structure–activity relationship should be noted regarding the cholinesterase inhibitory data of Table 1: (i) All the synthesized compounds showed higher inhibitory activity against AChE in comparison with BuChE. (ii) Electron-withdrawing group (nitro) at the para-position of the phenyl ring exhibited higher inhibitory activity than electron-donating groups (methoxy and methyl) for both ChEs [compared **4k** (R = 4-NO_2, IC_{50} = 0.165 μM for AChE, IC_{50} = 0.443 μM for BuChE), with **4g** (R = 4-OCH_3, IC_{50} = 0.352 μM for AChE, IC_{50} = 1.822 μM for BuChE) and **4i** (R = 4-CH_3, IC_{50} = 0.350 μM for AChE, IC_{50} = 0.866 μM for BuChE)]. (iii) Moving the bromine atom at the phenyl ring from the meta-position to the para-position led to a significant increase in the AChE and BuChE inhibition [compared **4e** (R = 3-Br, IC_{50} = 0.307 μM and 2.219 μM for AChE and BuChE, respectively) with **4f** (R = 4-Br, IC_{50} = 0.274 μM and 1.443 μM for AChE and BuChE, respectively)]. (iv) The presence of chlorine atom at the meta-position or para-position or both positions of the phenyl ring did not change the AChE activity (compared **4b** (R = 3-Cl, IC_{50} = 0.248 μM) with **4c** (R = 4-Cl, IC_{50} = 0.250 μM) and **4d** (R = 3,4-diCl, IC_{50} = 0.222 μM). On the other hand, moving the chlorine atom at the phenyl ring from the meta-position to the para-position dramatically decreased the BuChE activity (compared **4b** (R = 3-Cl, IC_{50} = 0.640 μM) with **4c** (R = 4-Cl, IC_{50} = 4.881 μM) and **4d** (R = 3,4-diCl, IC_{50} = 4.796 μM)). (v) The increase in the number of methoxy groups at the phenyl ring led to a decrease in the AChE inhibitory activity, whereas it caused a significant increase in the BuChE activity [compared **4g** (R = 4-OCH_3, IC_{50} = 0.352 μM and 1.822 μM for AChE and BuChE, respectively) with **4h** (R = 2,3- diOCH_3, IC_{50} = 0.510 μM and 0.182 μM for AChE and BuChE, respectively)]. (vi) Growing size and polarizability of the halogens at the *para*-position of the phenyl ring decreased the AChE inhibition [for size and polarizability, Br > Cl > F, for inhibitory activity, **4a** (R = 4-F, IC_{50} = 0.091 μM) > **4c** (R = 4- Cl, IC_{50} = 0.250 μM) > **4f** (R = 4-Br, IC_{50} = 0.274 μM)].

Cell toxicity

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity method was applied for cell viability test [33]. The per cent inhibitions of the synthesized compounds at different concentrations against the HepG2 cell line are summarized in Table 2. All tested compounds were compared with tacrine, and their inhibition values were lower than or equal to the tacrine (Table 2). In the presence of **4a** and **4h** did not significantly change the viability percentage of HepG2 cells at a concentration of 100 μM, and this value decreased to 54.52% in the presence of tacrine at the same concentration. If the values given in Table 2 are examined, it

Table 1 In vitro inhibition IC_{50} values (μM) of **4a–4k** and **8a–8c** for AChE and BuChE



Compound	R	AChE (IC_{50}, μM)[a]	BuChE (IC_{50}, μM)[a]
4a	4-F-phenyl	0.091 ± 0.022	0.773 ± 0.201
4b	3-Cl-phenyl	0.248 ± 0.102	0.640 ± 0.044
4c	4-Cl-phenyl	0.250 ± 0.111	4.881 ± 0.112
4d	3,4-diCl-phenyl	0.222 ± 0.021	4.796 ± 0.124
4e	3-Br-phenyl	0.307 ± 0.033	2.219 ± 0.142
4f	4-Br-phenyl	0.274 ± 0.054	1.443 ± 0.077
4g	4-OCH_3-phenyl	0.352 ± 0.031	1.822 ± 0.085
4h	2,3-diOCH_3-phenyl	0.510 ± 0.032	0.182 ± 0.012
4i	4-CH_3-phenyl	0.350 ± 0.103	0.866 ± 0.052
4j	Phenyl	1.197 ± 0.046	1.679 ± 0.035
4k	4-NO_2-phenyl	0.165 ± 0.055	0.443 ± 0.026
8a	3-Pyridinyl	0.358 ± 0.063	0.836 ± 0.045
8b	3-Benzo[b]thiophenyl	0.298 ± 0.041	1.656 ± 0.051
8c	2-(5-Methylfuranyl)	0.533 ± 0.074	0.521 ± 0.105
Tacrine	–	0.055 ± 0.002	0.032 ± 0.002
Galantamine	–	1.054 ± 0.022	18.130 ± 1.003
Donepezil	–	0.101 ± 0.052	2.680 ± 0.376

[a]IC_{50} values represent the mean ± SEM of three parallel measurements ($p < 0.05$)

Table 2 Effect of the synthesized compounds on the viability of HepG2 cell line at different concentrations

Compound	Viability (%) of HepG2 cells							
	100 μM	50 μM	30 μM	20 μM	10 μM	5 μM	3 μM	1 μM
4a	84.38 ± 1.2*	94.96 ± 1.3	97.30 ± 1.1	98.66 ± 1.4	99.59 ± 0.2	99.75 ± 0.6	99.06 ± 1.6	99.13 ± 1.9
4b	39.54 ± 2.1	73.11 ± 0.5	90.13 ± 1.5	91.60 ± 0.9	94.67 ± 0.6	96.60 ± 0.7	98.29 ± 0.9	99.29 ± 0.8
4c	55.18 ± 0.7	68.74 ± 0.8	86.45 ± 2.0	87.60 ± 0.9	88.76 ± 0.7	92.66 ± 0.7	94.45 ± 0.5	96.44 ± 0.8
4d	37.01 ± 1.1	58.17 ± 1.2	77.25 ± 1.4	80.93 ± 1.4	89.82 ± 0.6	90.44 ± 0.5	91.60 ± 0.8	95.22 ± 0.6
4e	65.06 ± 1.3	84.22 ± 1.1	84.38 ± 0.7	86.38 ± 1.3	88.29 ± 0.4	89.20 ± 0.8	92.06 ± 1.2	94.59 ± 1.5
4f	48.74 ± 0.8	64.83 ± 1.4	84.61 ± 0.4	86.14 ± 0.5	87.05 ± 0.8	91.83 ± 1.2	95.65 ± 1.1	97.03 ± 1.7
4 g	52.19 ± 0.8	72.42 ± 0.4	73.80 ± 0.8	77.02 ± 1.1	81.39 ± 0.4	84.61 ± 2.1	88.52 ± 1.6	87.14 ± 0.8
4 h	74.03 ± 0.4	79.78 ± 0.8	84.99 ± 1.1	85.61 ± 1.0	86.68 ± 0.8	86.22 ± 2.0	89.90 ± 0.5	93.12 ± 1.8
4i	53.34 ± 1.3	62.30 ± 1.2	78.17 ± 1.3	80.47 ± 1.2	87.60 ± 1.0	87.37 ± 1.8	91.74 ± 0.4	95.42 ± 1.1
4j	88.75 ± 1.2	91.97 ± 1.8	91.51 ± 0.6	91.51 ± 0.7	91.90 ± 1.1	91.11 ± 1.4	91.28 ± 0.7	91.97 ± 0.4
4 k	86.91 ± 1.3	87.47 ± 0.9	88.51 ± 0.6	90.76 ± 0.2	90.68 ± 1.3	91.81 ± 1.3	93.67 ± 1.8	99.33 ± 2.0
8a	74.03 ± 1.1	81.16 ± 2.1	85.53 ± 0.9	87.60 ± 0.4	88.85 ± 0.9	90.13 ± 1.2	92.43 ± 2.0	96.80 ± 1.4
8b	49.66 ± 1.4	60.46 ± 1.0	73.11 ± 0.8	74.03 ± 1.1	78.63 ± 0.5	78.63 ± 1.0	79.32 ± 0.4	81.62 ± 0.7
8c	60.46 ± 0.5	79.31 ± 2.0	82.09 ± 1.6	82.15 ± 0.3	84.31 ± 0.5	89.36 ± 0.8	90.44 ± 0.5	94.50 ± 0.8
Tacrine	54.52 ± 0.6	66.83 ± 0.8	77.28 ± 0.9	81.49 ± 0.6	93.35 ± 0.4	94.62 ± 0.5	95.76 ± 0.7	97.35 ± 0.7

Mean ± SEM of triplicates from at least three different cultures
*$p < 0.05$, as compared to the control cultures (one-way ANOVA)

Table 3 Log *P* values of the synthesized naphthyridine-11-amine derivatives

Compound	Log P^a
4a	5.75 ± 0.64
4b	6.38 ± 0.54
4c	6.38 ± 0.54
4d	6.83 ± 0.56
4e	6.49 ± 0.63
4f	6.72 ± 0.62
4 g	5.63 ± 0.55
4 h	5.15 ± 0.56
4i	6.27 ± 0.53
4j	5.81 ± 0.53
4 k	5.43 ± 0.55
8a	4.50 ± 0.54
8b	7.98 ± 0.58
8c	5.47 ± 0.63
Tacrine	3.32 ± 0.25

[a]Log *P* calculated from ChemSketch ACD labs 2012

will be seen that the selected compounds showed less toxicity on HepG2 cells compared to tacrine.

The log *P* value, known as the octanol/water partition coefficient, is one of the most important physicochemical parameters applied, especially when new drugs are developed. Log *P* is one of the "5 rules" defined by Lipinski for drug-like molecules and is therefore used as a physicochemical parameter in drug discovery studies related to the bioavailability of chemical compounds [34]. The ACD/ChemSketch software (ACD/ChemSketch 4.0) estimates physicochemical properties using atomic or group differences in chemical structure [35]. The log *P* values of the synthesized compounds and tacrine were calculated using the ACD/ChemSketch program. The results are given in Table 3. According to these results, the distribution coefficients of the synthesized compounds are better than tacrine.

In vitro blood–brain barrier permeability using PAMPA-BBB

A good penetration across the blood–brain barrier is the necessary condition for the central nervous system (CNS) drugs [36]. Brain permeations of **4a**, the most potent compound in this study, and the well-known AChE inhibitors (tacrine, donepezil and rivastigmine) were determined through the parallel artificial membrane permeation assay (PAMPA), described by Di et al. [37]. This assay measures the passive diffusion of a compound into an acceptor chamber filled with phosphate buffer (pH 7.4) through a lipid barrier separating a donor compartment. The concentrations of the compound in both partitions were then determined to obtain an effective permeability ratio (P_e). It is known that compounds with P_e values of 4×10^{-6} cm s^{-1} can easily pass through the CNS (CNS+) and compounds with a P_e value below 2×10^{-6} cm s^{-1} cannot pass through the CNS (CNS−). In compounds with permeability values between these boundaries, it is not easy to predict whether they pass through the BBB (CNS +/−) [38, 39]. **4a** has a permeability value above the boundary with $P_e = 5.42 \times 10^{-6}$ cm s^{-1} and indicates that it will pass through BBB with passive diffusion (Table 4). Donepezil ($P_e = 6.80 \times 10^{-6}$ cm s^{-1}) showed a higher P_e than **4a**; the P_e of Rivastigmine ($P_e = 5.78 \times 10^{-6}$ cm s^{-1}) was fairly close to 4a, while the P_e of tacrine ($P_e = 4.51 \times 10^{-6}$ cm s^{-1}) showed a less P_e than **4a**.

Table 4 Prediction of blood–brain barrier penetration of drugs expressed as $P_e \pm$ SEM ($n = 4$–6)

Compound	BBB penetration estimation	
	P_e (10^{-6} cm s^{-1})	CNS (+/−)
4a	5.42 ± 0.54	CNS (+)
Tacrine	4.51 ± 0.32	CNS (+)
Donepezil	6.80 ± 0.66	CNS (+)
Rivastigmine	5.78 ± 0.85	CNS (+)

'CNS(+)'—high BBB permeation predicted; P_e (10^{-6} cm s^{-1}) > 4.0
'CNS(−)'—low BBB permeation predicted; P_e (10^{-6} cm s^{-1}) < 2.0
'CNS(+/−)'—BBB permeation uncertain; P_e (10^{-6} cm s^{-1}) from 4.0 to 2.0

Conclusions

A series of 14 novel tacrine-based naphthyridine-11-amine derivatives (**4a–4k** and **8a–8c**) were synthesized, and their inhibitory activities on AChE and BuChE were evaluated. Most of the compounds showed potent activity against cholinesterase enzyme. Among them, compound **4a** showed the strongest inhibition against AChE with an IC_{50} value of 0.091 μM and **4h** showed the strongest inhibition against BuChE with an IC_{50} value of 0.138 μM being more potent than reference drug tacrine. Furthermore, **4a** showed less cytotoxicity on HepG2 cells compared to tacrine. The structure–activity relationship (SAR) for the synthesized compounds is indicated by comparing the effects of different groups and atoms on the naphthyridine skeleton. The SAR study revealed that electron-withdrawing and electron-releasing groups at different positions could increase the cholinesterase inhibition. According to the results of parallel artificial membrane permeability test (PAMPA-BBB), the permeability value of **4a** ($P_e = 5.42 \times 10^{-6}$ cm s^{-1}) is higher than the border, and this molecule is thought to pass through BBB with passive diffusion. In general, the activity of these compounds can be enhanced by applying more appropriate substitution patterns and presented as novel precursor com-

pounds for the development of new ChEs inhibitors for the treatment of AD.

Experimental

Material and method

All solvents, reagents and starting materials were obtained from commercial sources unless otherwise indicated. Melting points were measured on a Stuart SMP40. IR spectra were registered on a Bruker Alpha infrared spectrometer. ^{1}H and ^{13}C NMR spectra were registered on a Varian Infinity Plus spectrometer at 300 and at 75 Hz, respectively. ^{1}H and ^{13}C chemical shifts are referenced to the internal deuterated solvent. Mass spectra were obtained using Zivak Technologies LC–MS spectrometry. The elemental analyses were carried out with a Leco CHNS-932 instrument. Spectrophotometric analyses were performed by a BioTek Power Wave XS (BioTek, USA). The electric eel acetylcholinesterase (AChE, Type-VI-S, EC 3.1.1.7, 425.84 U/mg, Sigma) and horse serum butyrylcholinesterase (BuChE, EC 3.1.1.8, 11.4 U/mg, Sigma) were purchased from Sigma (Steinheim, Germany). The other chemicals and solvents were purchased from Fluka Chemie, Merck, Alfa Aesar and Sigma-Aldrich.

General procedures of synthesis and spectral data

Synthesis of malononitrile derivatives (2a–k and 6a–c) A mixture of aldehyde (0.01 mmol) and malononitrile (0.01 mmol) in ethanol (15 mL) in the presence of piperidine (0.5 eq) was warmed at 80 °C until complete precipitation (reaction times 1 h). The solid obtained was collected by filtration and recrystallized from ethanol and dried, to give compounds in good yield.

2-(4-Fluorobenzylidene)malononitrile (2a) Yellow powder, 62% yield; IR: 3032, 2224, 1580, 1553, 1485, 1406, 1214, 1093, 935, 826, 627 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ/ppm: 7.20–7.26 (2H, m), 7.75 (1H, s), 7.93–7.98 (2H, m);^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 82.2, 112.7 (2C), 113.8, 117.6 (2C) (d, $Jo_{C,F} = 22.3$ Hz), 127.6 (d, $Jp_{C,F} = 3.2$ Hz), 133.6(2C)(d, $Jm_{C,F} = 9.5$ Hz), 164.6, 168.0.

2-(3-Chlorobenzylidene)malononitrile (2b) White powder, 78% yield; IR: 3030, 2220, 1582, 1555, 1485, 1406, 1210, 1083, 936, 825, 625 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ/ppm: 7.46–7.52 (1H, m), 7.58–7.61 (1H, m), 7.72 (1H, s), 7.82–7.85 (2H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 84.4, 112.2 (2C), 113.4, 128.5, 131.1, 132.5, 134.6, 136.0, 158.4.

2-(4-Chlorobenzylidene)malononitrile (2c) Yellow powder, 80% yield; IR: 3032, 2224, 1582, 1555, 1485, 1406, 1210, 1094, 935, 825, 627 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ/ppm: 7.52 (2H, d, $J = 8.7$ Hz), 7.83 (1H, s), 7.86 (2H, d, $J = 8.7$ Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 83.5, 112.5 (2C), 129.4 (2C), 130.3 (2C), 132.0, 141.4, 158.5.

2-(3,4-Dichlorobenzylidene)malononitrile (2d) Cream powder, 76% yield; IR: 3034, 2223, 1581, 1550, 1484, 1403, 1208, 1090, 933, 822, 625 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ/ppm: 7.62 (1H, d, $J = 8.4$ Hz), 7.69 (1H, s), 7.7.79–7.82 (1H, m), 7.93 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 85.0, 112.1(2C), 129.2, 130.6, 131.9, 132.5, 134.5, 139.4, 157.2.

2-(3-Bromobenzylidene)malononitrile (2e) Cream powder, 62% yield; IR: 3040, 2198, 1592, 1432, 1212, 1076, 1041, 876,752 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ/ppm: 7.54–7.59 (1H, m), 7.86–7.94 (2H, m), 8.07 (1H, s), 8.51 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 84.2, 113.5 (2C), 123.0, 129.6, 132.7, 133.5, 134.0, 137.2, 160.5.

2-(4-Bromobenzylidene)malononitrile (2f) Yellow powder, 78% yield; IR: 3038, 2200, 1590, 1432, 1210, 1076, 1041, 876, 752 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ/ppm: 7.86 (4H, s), 8.53 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 82.9, 113.7 (2C), 114.7, 129.0 (2C), 131.0 (2C), 132.8, 133.3, 160.9.

2-(4-Methoxybenzylidene)malononitrile (2g) Yellow powder, 80% yield; IR: 3030, 2219, 1603, 1555, 1509, 1367, 1275, 1176, 1019, 832 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ/ppm: 3.90 (3H, s), 7.00 (2H, d, $J = 9.0$ Hz), 7.65 (1H, s), 7.91 (2H, d, $J = 9.0$ Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 56.0, 78.6, 113.6 (2C), 114.7 (2C), 124.2, 133.7 (2C), 159.1, 165.0.

2-(2,3-Dimethoxybenzylidene)malononitrile (2h) Yellow powder, 82% yield; IR: 3030, 2220, 1603, 1555, 1509, 1367, 1275, 1176, 1019, 832 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ/ppm: 3.90 (3H, s), 3.93 (3H, s), 7.15–7.17 (2H, m), 7.78–7.81 (1H, m), 8.25 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 56.2, 62.3, 83.0, 112.9 (2C), 114.4, 118.6, 119.8, 124.8, 149.8, 152.9, 155.0.

2-(4-Methylbenzylidene)malononitrile (2i) White powder, 80% yield; IR: 3035, 2220, 1603, 1584, 1367, 1217, 938, 812 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ/ppm: 2.38 (3H, s), 7.40 (2H, d, $J = 8.2$ Hz), 7.83 (2H, d, $J = 8.2$ Hz), 8.44 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.1, 80.5, 114.1 (2C), 115.1 (2C), 129.4 (2C), 130.8, 131.3, 161.9.

2-Benzylidenemalononitrile (2j) Cream powder, 78% yield; IR: 3038, 2222, 1605, 1584, 1367, 1218, 938, 812 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ/ppm: 7.51–7.66 (3H, m), 7.78 (1H, s), 7.90 (2H, d, $J = 7.6$ Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 83.0, 112.8 (2C), 113.9, 129.8 (2C), 131.0 (2C), 131.1, 160.2.

2-(4-Nitrobenzylidene)malononitrile (2k) Light brown, 72% yield; IR: 3040, 2219, 1603, 1558, 1509, 1367, 1275, 1176, 1019, 832 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 8.12 (2H, d, J = 8.4 Hz), 8.41 (2H, d, J = 8.2 Hz), 8.70 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 86.6, 113.2 (2C), 125.0 (2C), 132.1 (2C), 137.3, 150.3, 159.9.

2-(Pyridin-3-yl-methylene)malononitrile (6a) Brown powder, 76% yield; IR: 3038, 2220, 1605, 1584, 1510, 1367, 1280, 1218, 938, 812 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 7.62–7.67 (1H, m), 8.36 (1H, d, J = 8.2 Hz), 8.61 (1H, s), 8.78 (1H, d, J = 6.1 Hz), 8.96 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 84.7, 113.5 (2C), 125.0, 128.2, 136.9, 152.2, 154.7, 159.6.

2-(Benzo[b]thiophen-3-yl-methylene)malononitrile (6b) Yellow powder, 84% yield; IR: 3040, 2218, 1603, 1558, 1509, 1367, 1280, 1174, 1032, 837 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 7.34–7.44 (2H, m), 7.77–7.82 (2H, m), 8.00 (1H, s), 8.77 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 81.1, 113.8 (2C), 120.9, 123.2, 126.2, 126.4, 127.7, 136.1, 137.2, 139.1, 149.1.

2-((5-Methylfuran-2-yl)methylene)malononitrile (6c) Brown powder, 80% yield; IR: 3036, 2219, 1605, 1558, 1509, 1367, 1280, 1174, 1090, 837 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 2.42 (3H, s), 6.59 (1H, d, J = 3.8 Hz), 7.37 (1H, d, J = 3.5 Hz), 8.13 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 14.7, 72.8, 112.9, 114.3, 115.6, 128.8, 144.1, 147.7, 162.9.

Synthesis of aminocyanopyridine derivatives (3a–k and 7a–c) Compounds **2a–k** and **6a–c** (5.0 mmol) were suspended in benzene (60 mL), and ammonium acetate (7.5 mmol) and cycloalkane 4 (5.0 mmol) were added. The flask was fitted with a reflux condenser and a water separator. The mixture was refluxed for 10 h. Then, the solvent was evaporated, and the mixture was redissolved in dichloromethane (150 mL) and washed with water (2 × 50 mL). Then, the organic phase was dried with $NaSO_4$, filtered off and recrystallized from ethanol. All aminocyanopyridines (**3a–k** and **7a–c**) were prepared by this procedure.

2-Amino-4-(4-fluorophenyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (3a) Yellow powder, 78% yield; IR: 3423, 3301, 3137, 2939, 2212, 1642, 1555, 1508, 1249, 1157, 844 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.62–1.69 (2H, m), 1.78–1.86 (2H, m), 2.30 (2H, t, J = 6.1 Hz), 2.80 (2H, t, J = 6.4 Hz), 5.20 (2H, s), 7.09–7.25 (4H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.6, 22.7, 26.7, 33.5, 90.2, 115.9 116.9(d, $Jo_{C,F}$ = 46.8 Hz), 121.0, 130.3 (d, $Jm_{C,F}$ = 8.3 Hz), 132.2 (d, $Jp_{C,F}$ = 3.3 Hz), 153.7, 157.3, 161.4, 161.8.

2-Amino-4-(3-chlorophenyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (3b) Yellow powder, 70% yield; IR: 3419, 3301, 3141, 2926, 2209, 1639, 1553, 1455, 1420, 1246, 1171, 1084, 772 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.64–1.71 (2H, m), 1.79–1.87 (2H, m), 2.30 (2H, t, J = 6.4 Hz), 2.80 (2H, t, J = 6.4 Hz), 5.13 (2H, s), 7.12–7.15 (1H, m), 7.25 (1H, s), 7.40–7.42 (2H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.6, 22.9, 26.6, 33.5, 89.9, 116.6, 120.8, 126.5, 128.3, 129.3, 130.4, 134.8, 138.0, 153.0, 157.2, 162.0.

2-Amino-4-(4-chlorophenyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (3c) Yellow powder, 78% yield; IR: 3420, 3301, 3141, 2926, 2212, 1639, 1553, 1455, 1420, 1246, 1171, 1080, 772 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.64–1.70 (2H, m), 1.78–1.84 (2H, m), 2.30 (2H, t, J = 6.1 Hz), 2.80 (2H, t, J = 6.1 Hz), 5.11 (2H, s), 7.19 (2H, d, J = 8.2 Hz), 7.45 (2H, d, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.5, 22.8, 26.4, 33.3, 89.3, 116.8, 120.1, 128.9, 129.7, 134.7, 134.8, 153.0, 157.5, 161.7.

2-Amino-4-(3,4-dichlorophenyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (3d) Yellow powder, 80% yield; IR: 3410, 3303, 3140, 2936, 2102, 1630, 1555, 1455, 1419, 1248, 1170, 1079, 775 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.48–1.51 (2H, m), 1.61–1.69 (2H, m), 2.13 (2H, t, J = 6.4 Hz), 2.62 (2H, t, J = 6.4 Hz), 5.35 (2H, s), 6.96 (1H, d, J = 8.2 Hz), 7.25 (1H, s), 7.40 (1H, d, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.4, 22.8, 26.4, 33.4, 89.1, 116.5, 120.1, 127.8, 130.2, 131.0, 132.9, 133.1, 136.3, 151.6, 157.5, 162.2.

2-Amino-4-(3-bromophenyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (3e) Brown powder, 70% yield; IR: 3416, 3302, 3138, 2924, 2198, 1640, 1553, 1456, 1420, 1248, 1180, 1080, 772 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.64–1.72 (2H, m), 1.78–1.86 (2H, m), 2.27–2.34 (2H, m), 2.80 (2H, t, J = 6.4 Hz), 5.18 (2H, s), 7.18 (1H, d, J = 8.2 Hz), 7.32–7.39 (2H, s), 7.57 (1H, d, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.6, 22.9, 26.6, 33.4, 89.9, 116.5, 120.7, 122.9, 127.0, 130.6, 131.1, 132.1, 138.3, 152.9, 157.4, 162.0.

2-Amino-4-(4-bromophenyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (3f) Yellow powder, 74% yield; IR: 3422, 3303, 3142, 2918, 2186, 1640, 1555, 1455, 1420, 1240, 1179, 1080, 776 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.64–1.71 (2H, m), 1.79–1.85 (2H, m), 2.31 (2H, t, J = 6.4 Hz), 2.81 (2H, t, J = 6.4 Hz), 5.09 (2H, s), 7.14 (2H, d, J = 8.4 Hz), 7.62 (2H, d, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.7, 23.0, 26.6, 33.5, 89.8, 116.7, 120.8, 123.5, 130.0, 131.0, 132.2, 132.7, 135.1, 153.3, 157.2, 162.0.

2-Amino-4-(4-methoxyphenyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (3g) White powder, 64% yield; IR: 3449, 3410, 3154, 2934, 2212, 1637, 1555,

1422, 1259, 1069, 1001, 743 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.63–1.69 (2H, m), 1.79–1.84 (2H, m), 2.35 (2H, t, J = 6.4 Hz), 2.79 (2H, t, J = 6.1 Hz), 3.84 (3H, s), 5.11 (2H, s), 6.98 (2H, d, J = 8.2 Hz), 7.19 (2H, d, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.8, 23.1, 26.8, 33.5, 55.5, 90.4, 114.2, 115.3, 117.2, 121.3, 128.4, 129.8, 133.7, 154.5, 157.3, 160.1, 161.5.

2-Amino-4-(2,3-dimethoxyphenyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (3h) Yellow powder, 60% yield; IR: 3452, 3408, 3150, 2930, 2210, 1635, 1555, 1420, 1259, 1079, 1001, 745 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.60–1.69 (2H, m), 1.77–1.85 (2H, m), 2.14–2.22 (1H, m), 2.31–2.38 (1H, m), 2.77–2.82 (2H, m), 3.69 (3H, s), 3.90 (3H, s), 5.11 (2H, s), 6.67 (1H, d, J = 7.6 Hz), 6.98 (1H, d, J = 8.2 Hz), 7.14 (1H, t, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.7, 22.8, 25.3, 33.5, 55.9, 61.3, 90.4, 113.1, 116.9, 121.0, 122.3, 124.8, 130.7, 145.8, 152.1, 153.1, 157.0, 161.2.

2-Amino-4-(p-tolyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (3i) Yellow powder, 76% yield; IR: 3427, 3292, 3140, 2936, 2209, 1638, 1552, 1513, 1455, 1423, 1250, 1168, 801, 774 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.63–1.68 (2H, m), 1.78–1.89 (2H, m), 2.34 (2H, t, J = 6.4 Hz), 2.40 (3H, s), 2.79 (2H, t, J = 6.4 Hz), 5.18 (2H, s), 7.13 (2H, d, J = 7.9 Hz), 7.26 (2H, d, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 21.6, 22.7, 23.0, 26.7, 33.5, 90.3, 117.1, 121.1, 128.2, 129.6, 133.3, 139.0, 154.9, 157.3, 161.5.

2-Amino-4-phenyl-5,6,7,8-tetrahydroquinoline-3-carbonitrile (3j) Yellow powder, 72% yield; IR: 3420, 3300, 3140, 2930, 2214, 1638, 1552, 1510, 1450, 1422, 1250, 1168, 803, 775 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.63–1.69 (2H, m), 1.78–1.86 (2H, m), 2.32 (2H, t, J = 6.4 Hz), 2.80 (2H, t, J = 6.1 Hz), 5.17 (2H, s), 7.22–7.25 (2H, m), 7.42–7.49 (3H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.7, 23.0, 26.7, 33.5, 90.2, 116.9, 121.0, 128.3, 128.9, 129.0, 136.3, 154.7, 157.3, 161.6.

2-Amino-4-(4-nitrophenyl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (3k) Brown powder, 66% yield; IR: 3422, 3400, 3150, 2928, 2210, 1640, 1555, 1510, 1450, 1250, 1168, 873 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.65–1.69 (2H, m), 1.79–1.89 (2H, m), 2.24 (2H, t, J = 6.4 Hz), 2.76 (2H, t, J = 6.4 Hz), 5.35 (2H, s), 7.26 (2H, d, J = 8.4 Hz), 8.16 (2H, d, J = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 24.3, 26.4, 33.4, 35.8, 88.7, 112.6, 124.0, 124.2, 129.6, 130.7, 140.8, 143.1, 148.0, 151.8.

2-Amino-4-(pyridin-3-yl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (7a) Yellow powder, 60% yield; IR: 3418, 3336, 3142, 2938, 2210, 1639, 1554, 1420, 1240, 1168, 801, 729 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.55–1.62 (2H, m), 1.70–1.79 (2H, m), 2.18 (2H, t, J = 6.1 Hz), 2.69 (2H, t, J = 6.1 Hz), 6.65 (2H, s), 7.39–7.43 (1H, m), 7.76–7.81 (1H, m), 8.57 (1H, s), 8.64–8.66 (1H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.6, 23.0, 26.4, 33.5, 88.6, 114.5, 117.1, 119.1, 124.2, 132.9, 137.7, 149.8, 151.1, 158.6, 162.0.

2-Amino-4-(benzo[b]thiophen-3-yl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (7b) Cream powder, 70% yield; IR: 3420, 3300, 3140, 2940, 2212, 1642, 1552, 1419, 1218, 1168, 829, 729 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.59–1.66 (2H, m), 1.80–1.86 (2H, m), 2.25–2.29 (2H, m), 2.83–2.88 (2H, m), 5.23 (2H, s), 7.34–7.44 (1H, m), 7.92–7.94 (4H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.7, 22.8, 26.1, 33.4, 91.2, 116.5, 122.5, 122.6, 123.2, 124.9, 125.1, 126.1, 131.7, 137.2, 140.3, 149.1, 157.3, 161.6.

2-Amino-4-(5-methylfuran-2-yl)-5,6,7,8-tetrahydroquinoline-3-carbonitrile (7c) Red powder, 40% yield; IR: 3418, 3360, 3141, 2946, 2210, 1638, 1550, 1422, 1212, 1160, 825 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.68–1.80 (2H, m), 1.82–1.87 (2H, m), 2.39 (3H, s), 2.68 (2H, t, J = 6.1 Hz), 2.78 (2H, t, J = 6.4 Hz), 5.16 (2H, s), 6.15 (1H, d, J = 5.4 Hz),6.75 (1H, d, J = 5.5 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 13.9, 22.5, 23.0, 27.2, 33.7, 86.8, 108.1, 115.8, 117.8, 120.0, 141.6, 146.4, 154.5, 158.0, 161.7.

General procedure for the of compounds 4a–k and 8a–c The 2-amino-4-phenyl-5,6,7,8-tetrahydroquinoline-3-carbonitrile derivatives (1.0 eq) and $ZnCl_2$ (1.5 eq) and cyclohexanone (12 eq) were mixed. The reaction mixture was heated at 140 °C for 4 h. When the reaction was complete, the reaction mixture was diluted with a solution of dichloromethane/water (1/1) and treated with an aqueous solution of sodium hydroxide (10%) until pH 11–12. After stirring for 30 min, the mixture was extracted with CH_2Cl_2, dried over anhydrous sodium sulphate, filtered and the solvent was evaporated. The solid obtained washed with ether and filtered gives the pure product.

12-(4-Fluorophenyl)-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1,8]naphthyridin-11-amine (4a) Light brown powder, 50% yield; mp. 170–172 °C; IR: 3490, 3410, 2931, 2864, 1614, 1562, 1543, 1505, 1427, 1328, 1218, 1093, 854 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.70–1.72 (2H, m), 1.85 (6H, s, br), 2.28–2.30 (4H, m), 2.99 (2H, s, br), 3.09–3.13 (2H, m), 4.10 (2H, s), 7.19–7.26 (4H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.8, 23.0, 23.3, 23.9, 27.8, 34.3, 108.1, 111.0, 116.6 (d, $Jo_{,F}$ = 21.4 Hz), 127.5, 130.4(d, $Jm_{C,F}$ = 7.8 Hz), 135.5(d, $Jp_{C,F}$ = 3.8 Hz), 144.1, 148.2, 153.6, 160.7, 160.8, 164.4. LC–MS (m/z): 348.1 $[MH]^+$. Anal. Calcd. for $C_{22}H_{22}FN_3$: C, 76.05; H, 6.38; N, 12.09; found: C, 76.12; H, 6.42; N, 12.25.

12-(3-Chlorophenyl)-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1,8]naphthyridin-11-amine (4b)
Cream powder, 60% yield; mp. 225–227 °C; IR: 3440, 3380, 2937, 2857, 1638, 1561, 1542, 1431, 1302, 1146, 1078, 800, 673 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.73–1.77 (2H, m), 1.85–1.89 (6H, m), 2.32–2.39 (4H, m), 3.01 (2H, t, *J* = 6.1 Hz), 3.12 (2H, t, *J* = 6.4 Hz), 4.10 (2H, s), 7.20 (1H, t, *J* = 4.1 Hz), 7.30 (1H, s), 7.47 (2H, d, *J* = 4.6 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.8, 23.0, 23.3, 23.9, 27.8, 34.3, 107.8, 111.1, 126.8, 127.1, 128.7, 130.9, 135.6, 141.6, 143.5, 148.1, 153.6, 160.9. LC–MS (m/z): 364.1 $[MH]^+$. Anal. Calcd. for $C_{22}H_{22}ClN_3$: C, 72.62; H, 6.09; N, 11.55; found: C, 72.50; H, 6.00; N, 11.78.

12-(4-Chlorophenyl)-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1,8]naphthyridin-11-amine *(4c)*
Light brown powder, 67% yield; mp. 142–145 °C; IR: 3430, 3400, 2930, 2857, 1625, 1563, 1541, 1491, 1428, 1304, 1088, 1015, 863 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.64–1.74 (2H, m), 1.80–2.01 (6H, m), 2.28–2.32 (4H, m), 2.99 (2H, t, *J* = 5.8 Hz), 3.10 (2H, t, *J* = 6.4 Hz), 4.13 (2H, s), 7.23 (2H, d, *J* = 8.2 Hz), 7.50 (2H, d, *J* = 7.9 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.8, 23.0, 23.3, 23.9, 25.2, 27.8, 34.3, 42.2, 107.9, 111.0, 123.8, 127.3, 129.8, 130.1, 134.8, 136.0, 138.1, 143.8, 148.2, 153.5, 160.7, 160.9. LC–MS (m/z): 364.0 $[MH]^+$. Anal. Calcd. for $C_{22}H_{22}ClN_3$: C, 72.62; H, 6.09; N, 11.55; found: C, 72.70; H, 6.12; N, 11.65.

12-(3,4-Dichlorophenyl)-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1,8]naphthyridin-11-amine (4d)
Brown powder, 67% yield; mp. 201–203 °C; IR: 3470, 3380, 2931, 2857, 1632, 1563, 1541, 1433, 1315, 1135, 1031, 938, 822 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.71–1.77 (2H, m), 1.85–1.88 (6H, m), 2.26–2.39 (4H, m), 3.01 (2H, t, *J* = 5.8 Hz), 3.11 (2H, t, *J* = 6.1 Hz), 4.14 (2H, s), 7.17 (1H, dd, *J* = 8.7, 1.7 Hz), 7.40 (1H, s), 7.61 (1H, d, *J* = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.7, 22.9, 23.2, 23.9, 27.8, 34.2, 34.3, 107.7, 111.3, 127.3, 128.1, 130.6, 131.6, 133.2, 134.0, 139.6, 142.4, 148.0, 153.4, 160.9, 161.0. LC–MS (m/z): 398.0 $[MH]^+$. Anal. Calcd. for $C_{22}H_{21}Cl_2N_3$: C, 66.34; H, 5.31; N, 10.55; found: C, 66.42 H, 5.55; N, 10.62.

12-(3-Bromophenyl)-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1,8]naphthyridin-11-amine (4e)
Light brown powder, 57% yield; mp. 174–176 °C; IR: 3420, 3380, 2932, 2858, 1618, 1563, 1543, 1431, 1326, 1169, 1143, 1071, 967, 693 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.61–1.74 (4H, m), 1.85–2.13 (6H, m), 2.30–2.35 (4H, m), 3.04–3.09 (2H, m), 4.58 (2H, s), 7.19–7.25 (1H, m), 7.40–7.45 (2H, m), 7.64 (1H, d, *J* = 7.9 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.3, 22.6, 23.1, 23.7, 27.7, 32.6, 34.2, 107.4, 111.1, 123.9, 127.1, 128.1, 131.4, 132.2, 140.9, 144.0, 150.2, 151.5, 159.0, 162.1. LC–MS (m/z): 408.1 $[MH]^+$. Anal. Calcd. for $C_{22}H_{22}BrN_3$: C, 64.71; H, 5.43; N, 10.29; found: C, 64.88; H, 5.56; N, 10.34.

12-(4-Bromophenyl)-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1,8]naphthyridin-11-amine (4f)
Light brown powder, 40% yield; mp. 227–229 °C; IR: 3480, 3410, 2930, 2860, 1698, 1608, 1565, 1542, 1426, 1313, 1069, 1011, 929, 834 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.69–1.77 (2H, m), 1.84–1.91 (6H, m), 2.30–2.34 (4H, m), 3.01 (2H, t, *J* = 5.8 Hz), 3.12 (2H, t, *J* = 6.4 Hz), 4.15 (2H, s), 7.20 (2H, d, *J* = 8.4 Hz), 7.67 (2H, d, *J* = 8.4 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.8, 23.0, 23.3, 23.9, 27.8, 34.2, 34.3, 107.8, 111.0, 122.9, 127.2, 130.3, 132.8, 138.5, 143.9, 148.2, 153.4, 160.7, 160.9. LC–MS (m/z): 408.1 $[MH]^+$. Anal. Calcd. for $C_{22}H_{22}BrN_3$: C, 64.71; H, 5.43; N, 10.29; found: C, 64.82; H, 5.60; N, 10.40.

12-(4-Methoxyphenyl)-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1,8]naphthyridin-11-amine (4g)
Yellow powder, 86% yield; mp. 209–211 °C; IR: 3460, 3400, 2928, 2857, 1633, 1563, 1541, 1510, 1435, 1284, 1243, 1177, 1030, 930, 835 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.67–1.75 (2H, m), 1.82–1.84 (6H, m), 2.31–2.35 (4H, m), 2.98 (2H, s, br), 3.09 (2H, t, *J* = 6.4 Hz), 3.86 (3H, s), 4.26 (2H, s), 7.03 (2H, d, *J* = 8.7 Hz), 7.16 (2H, d, *J* = 8.4 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.8, 23.0, 23.4, 23.8, 27.7, 34.1, 34.3, 55.5, 108.4, 110.7, 115.0, 127.9, 129.7, 131.3, 145.1, 148.7, 153.5, 159.7, 160.4, 160.8. LC–MS (m/z): 360.2 $[MH]^+$. Anal. Calcd. for $C_{23}H_{25}N_3O$: C, 76.85; H, 7.01; N, 11.69; found: C, 77.00; H, 7.21; N, 11.52.

12-(2,3-Dimethoxyphenyl)-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1,8]naphthyridin-11-amine(4h)
Light brown powder, 72% yield; mp. 162–164 °C; IR: 3480, 3410, 2938, 2856, 1622, 1558, 1540, 1512, 1436, 1284,, 1277, 1030, 930, 837 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.67–1.76 (2H, m), 1.80–1.84 (6H, m), 2.24–2.38 (4H, m), 2.99 (2H, s, br), 3.10 (2H, t, *J* = 6.4 Hz), 3.59 (3H, s), 3.92 (3H, s), 4.32 (2H, s), 6.68 (1H, d, *J* = 7.9 Hz), 7.08 (2H, d, *J* = 7.9 Hz), 7.18 (1H, t, *J* = 8.2 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.8, 22.9, 23.0, 23.3, 23.9, 27.2, 27.4, 34.3, 56.0, 60.0, 108.5, 110.7, 112.9, 121.1, 125.3, 127.7, 133.7, 141.7, 145.8, 148.5, 153.6, 160.4, 160.8. LC–MS (m/z): 390.2 $[MH]^+$. Anal. Calcd. for $C_{24}H_{27}N_3O_2$: C, 74.01; H, 6.99; N, 10.79; found: C, 74.66; H, 7.20; N, 11.00.

12-(p-Tolyl)-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1,8] naphthyridin-11-amine (4i) Light brown powder, 64% yield; mp. 193–195 °C; IR: 3490, 3280, 2929, 2857, 1633, 1567, 1541, 1512, 1430, 1314, 1138, 1110, 930, 813 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.63–1.76 (2H, m), 1.83–2.01 (6H, m), 2.22–2.39 (4H, m), 2.43 (3H, s), 2.99–3.01 (2H, m), 3.11 (2H, t, *J* = 6.4 Hz), 4.17 (2H, s), 7.14 (2H, d, *J* = 7.6 Hz), 7.31 (2H, d, *J* = 7.3 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 21.5, 22.6, 23.1, 23.4, 23.9, 27.7, 32.0, 34.3, 108.3, 110.6, 127.4, 128.4, 130.2, 136.6, 138.5, 145.3, 148.6, 153.6, 160.5, 160.7. LC–MS (m/z): 344.1 $[MH]^+$. Anal. Calcd. for $C_{23}H_{25}N_3$: C, 80.43; H, 7.34; N, 12.23; found: C, 80.62; H, 7.40; N, 12.36.

12-Phenyl-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1,8] naphthyridin-11-amine (4j) Light brown powder, 47% yield; mp. 146–148 °C; IR: 3440, 3280, 2929, 2858, 1619, 1564, 1544, 1433, 1325, 1217, 1138, 1119, 702 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.62–1.76 (2H, m), 1.85–2.00 (6H, m), 2.22–2.33(4H, m), 2.99–3.01 (2H, m), 3.10–3.12 (2H, m), 4.17 (2H, s), 7.25–7.37 (2H, m), 7.45–7.54 (3H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.7, 23.0, 23.4, 23.8, 27.7, 32.0, 34.3, 108.0, 111.6, 123.7, 127.3, 128.1, 131.2, 136.6, 138.5, 145.3, 153.7, 160.1, 160.8. LC–MS (m/z): 330.2 $[MH]^+$. Anal. Calcd. for $C_{22}H_{23}N_3$: C, 80.21; H, 7.04; N, 12.76; found: C, 80.36; H, 7.20; N, 12.80.

12-(4-Nitrophenyl)-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1,8]naphthyridin-11-amine(4k) Light brown powder, 62% yield; mp. 170–171 °C; IR: 3438, 3282, 2934, 2860, 1622, 1584, 1516, 1439, 1344, 1217, 1138, 1107, 847 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.65–1.74 (2H, m), 1.80–1.87 (6H, m), 2.24–2.39 (4H, m), 2.99–3.01 (2H, m), 3.04 (2H, t, *J* = 6.4 Hz), 4.20 (2H, s), 7.73 (2H, d, *J* = 8.7 Hz), 8.47 (2H, d, *J* = 8.7 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.6, 23.1, 23.4, 23.8, 27.8, 32.0, 34.3, 106.7, 111.3, 125.6, 127.3, 130.7, 136.6, 138.5, 144.2, 148.6, 153.7, 160.1, 160.8. LC–MS (m/z): 375.2 $[MH]^+$. Anal. Calcd. for $C_{22}H_{22}N_4O_2$: C, 70.57; H, 5.92; N, 14.96; found: C, 71.60; H, 6.22; N, 15.14.

12-(Pyridin-3-yl)-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1,8]naphthyridin-11-amine (8a) Red powder, 52% yield; mp. 171–173 °C; IR: 3460, 3400, 2932, 2860, 1606, 1566, 1544, 1483, 1427, 1316, 1251, 1093, 930, 711 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.66–1.75 (2H, m), 1.83–1.89 (6H, m), 2.24–2.35 (4H, m), 2.97–2.99 (2H, m), 3.11 (2H, t, *J* = 3.4 Hz),4.00 (2H, s), 7.44–7.49 (1H, m), 7.63–7.67 (1H, m), 8.55–8.56 (1H, m), 8.73 (1H, dd, *J* = 4.6, 1.4 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.7, 22.9, 23.2, 23.9, 28.1, 34.2, 34.3, 108.1, 111.4, 124.1, 127.7, 135.6, 136.4, 141.2, 148.0, 149.2, 150.0, 153.4, 161.0. LC–MS (m/z): 331.1 $[MH]^+$. Anal. Calcd. for $C_{21}H_{22}N_4$: C, 76.33; H, 6.71; N, 16.96; found: C, 76.52; H, 6.82; N, 16.90.

12-(Benzo[b]thiophen-3-yl)-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1,8]naphthyridin-11-amine (8b) Yellow powder, 83% yield; mp. 167–169 °C; IR: 3420, 3360, 2933, 2859, 1605, 1587, 1566, 1430, 1344, 1269, 1170, 1077, 1045, 830, 767 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.58–1.72 (2H, m), 1.82–2.00 (6H, m), 2.07–2.86 (4H, m), 3.10 (2H, t, *J* = 5.8 Hz), 3.21 (2H, s, br), 4.20 (2H, s), 7.14 (1H, d, *J* = 7.9 Hz), 7.30–7.48 (3H, m), 7.96 (1H, d, *J* = 7.9 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 22.5, 22.7, 23.5, 26.1, 26.7, 29.7, 32.0, 34.1, 107.9, 111.2, 122.4, 123.4, 125.7, 126.1, 131.9, 136.6, 140.5, 148.0, 149.1, 154.4, 155.0, 161.7, 164.3. LC–MS (m/z): 386.1 $[MH]^+$. Anal. Calcd. for $C_{24}H_{23}N_3S$: C, 74.77; H, 6.01; N, 10.90; found: C, 75.22; H, 6.42; N, 11.33.

12-(5-Methylfuran-2-yl)-1,2,3,4,7,8,9,10-octahydrodibenzo[b,g][1,8]naphthyridin-11-amine (8c) Red powder, 70% yield; mp. 99–101 °C; IR: 3430, 3340, 2929, 2859, 1633, 1588, 1434, 1372, 1207, 1022, 793 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ/ppm: 1.60–1.72 (2H, m), 1.82–2.30 (6H, m), 2.32 (3H, s), 2.34–2.86 (4H, m), 2.90–3.00 (2H, m), 3.20 (2H, s, br), 3.80 (2H, s), 6.17 (1H, s), 6.47 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ/ppm: 13.8, 22.4, 22.8, 23.1, 23.8, 28.3, 34.2, 34.3, 108.1, 111.7, 144.0, 133.6, 135.9, 143.6, 145.8, 152.6, 155.5, 156.3, 165.3. LC–MS (m/z): 334.1 $[MH]^+$. Anal. Calcd. for $C_{21}H_{23}N_3O$: C, 75.65; H, 6.95; N, 12.60; found: C, 75.90; H, 7.34; N, 12.85.

Biological activities

Anticholinesterase activity assays

Acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activities of the synthesized compounds were determined according to Ellman's method. The IC_{50} was determined by constructing an absorbance and/or inhibition (%) curve and examining the effect of five different concentrations. IC_{50} values were calculated for a given antagonist by determining the concentration needed to inhibit half of the maximum biological response of the agonist. The substrates of the reaction were acetylthiocholine iodide and butyrylthiocholine iodide. 5,5′-Dithiobis(2-nitrobenzoic) acid (DTNB) was used to measure anticholinesterase activity. Aliquots of 150 μL of 100 mM phosphate buffer (pH 8.0), 10 μL of sample solution and 20 μL AChE (2.476×10^{-4} U/μL) (or 3.1813×10^{-4} U/μL BuChE) solution were mixed and incubated for 15 min at 25 °C. 10 μL of DTNB solution was prepared by adding 2.0 mL of pH 7.0 and 4.0 mL of pH 8.0 phosphate buffers to a mixture of 1.0 mL of 16 mg/mL DTNB and 7.5 mg/mL

$NaHCO_3$ in pH 7.0 phosphate buffers. The reaction was initiated by the addition of 10 µL (7.1 mM) acetylthiocholine iodide (or 0.79 mM butyrylthiocholine iodide). In this method, the activity was measured by following the yellow colour produced as a result of the thiol anion produced by reacting the enzymatic hydrolysis of the substrate with DTNB. Also, methanol was used as a control solvent. The hydrolysis of the substrates was monitored using a BioTek Power Wave XS at 412 nm.

Cell cytotoxicity

The cytotoxicity effect of test compound on hepatocellular carcinoma (HepG2) cells was evaluated by MTT (3-(4,5 dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay according to described methods. Briefly, cells line were seeded in a flat-bottomed 96-well plate at a density of 5×10^4 cells/well in DMEM/RPMI containing 10% FBS. The plate was incubated at 37 °C with 5% CO_2 for 24 h, and then compounds were prepared and added to make a final concentration of 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.078 µM, respectively, in serum-free DMEM/RPMI. Cells were further incubated for 24 h at 37 °C with 5% CO_2; then, the medium was replaced with DMEM/RPMI containing 10% FBS. 10 µL of filter-sterilized MTT solution (5 mg/mL in PBS) was added to each well and further incubated at 37 °C with 5% CO_2 for 4 h. At the end of incubation, media was aspirated from the wells, and 100 µL of DMSO was added to dissolve insoluble Formosan crystals formed. The absorbance was measured at 570 nm using a microtiter plate reader. The relative % cell viability was calculated from the following equation: Relative per cent cell viability $= (A_{test}/A_{control}) \times 100\%$. ($A_{test}$ is the absorbance of the sample treated cells, and $A_{control}$ is the absorbance of the untreated cells. Each absorbance was taken to be the mean of triplicate measurements.) The cell viability was represented as a percentage relative to untreated cells as a control.

In vitro blood–brain barrier permeation assay

The Corning Gentest Pre-coated PAMPA Plate System (Cat. No. 353015) was used to perform permeability assays for novel compounds. In summary, the 96-well filter plate, pre-coated with lipids, was used as the permeation acceptor, and a matching 96-well receiver plate was used as the permeation donor. Compound solutions were prepared by diluting 10 mM DMSO stock solutions in PBS. (In most cases, we used a final concentration of 200 µM.) The compound solutions were added to the wells (300 µL/well) of the receiver plate, and PBS was added to the wells (200 µL/well) of the pre-coated filter plate. The filter plate was then coupled with the receiver plate, and the plate assembly was incubated at room temperature without agitation for five hours. At the end of the incubation, the plates were separated and 150 µL solution from each well of the filter plate and the receiver plate was transferred to UV transparent plates. The final concentrations of compounds in both donor wells and acceptor wells were analysed by a UV plate reader Synergy H1 (BioTek, USA). The concentration of the compound was calculated from the standard curve and expressed as permeability (P_e) by the following formula:

$$\text{Permeability (cm/s)}: \; P_e = \{-\ln[1 - C_A(t)/C_{eq}]\}/[A * (1/V_D + 1/V_A) * t]$$

A = filter area (0.3 cm^2), V_D = donor well volume (0.3 mL), V_A = acceptor well volume (0.2 mL), t = incubation time, $C_A(t)$ = compound concentration in acceptor well at time t, $C_D(t)$ = compound concentration in donor well at time t, and $C_{eq} = [C_D(t)*V_D + C_A(t)*V_A]/(V_D + V_A)$.

Supporting information summary

^1H NMR, ^{13}C NMR and MS spectra of the synthesized compounds are given in the Supporting Information.

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