Full Paper

Synthesis and Anticholinesterase Activity and Cytotoxicity of Novel Amide Derivatives

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In the present study, some amide derivatives were synthesized and their potential anticholinesterase properties were investigated. *N*-(Benzothiazol-2-yl)-2-[(5-amino/methyl-1,3,4-thiadiazol-2-yl)thio]acetamide derivatives were obtained by nucleophilic substitution of 2-chloro-*N*-(benzothiazole-2-yl)acetamide derivatives with appropriate 1,3,4-thiadiazole-2-thioles. The chemical structures of the compounds were elucidated by ^{1}H-NMR, ^{13}C-NMR and FAB^{+}-MS spectral data and elemental analyses. Each amide derivative was evaluated for its ability to inhibit AChE and BuChE using a modification of Ellman's spectrophotometric method. The compounds were also investigated for their cytotoxic properties using MTT assay. 2-(5-Amino-1,3,4-thiadiazol-2-yl)thio-*N*-(benzothiazol-2-yl)acetamide derivatives have anticholinesterase activity, whereas 2-(5-methyl-1,3,4-thiadiazol-2-yl)thio-*N*-(benzothiazol-2-yl)acetamide derivatives have no inhibitory effect on enzyme activity. Among these compounds, it is clear that compound **IIh** is the most potent derivative.

Keywords: Amide / Anticholinesterase activity / Cytotoxicity / Thiadiazole

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Introduction

Alzheimer's disease is the most common age-related neurodegenerative disorder characterized by progressive deficits in memory and cognition, together with impairment in the ability to perform basic activities of daily living. Alzheimer's disease has become an urgent public health problem due to the prevalence of Alzheimer's disease worldwide [1–9].

Over the last 20 years, scientists have carried out considerable research for deciphering the underlying mechanisms of this devastating disease. Substantial evidence demonstrates that Alzheimer's disease is associated with the deficiency in cholinergic neurotransmission and therefore current research interest in the design and synthesis of new cholinomimetics is focused on discovering agents that might be effective in the treatment of Alzheimer's disease [1–9].

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Acetylcholine, which is the physiologic cholinergic neurotransmitter, is a poor therapeutic agent due to its fleeting pharmacological action and chemical instability as a result of the rapid hydrolysis catalysed by cholinesterases [10].

Two cholinesterases are present in humans: acetylcholinesterase (AChE), which selectively hydrolyses acetylcholine, and butyrylcholinesterase (BuChE), which is a non-specific cholinesterase. The main difference between the two types of cholinesterase is the respective preferences for substrates: the former hydrolyses acetylcholine more quickly; the latter hydrolyses butyrylcholine more quickly. The main function of acetylcholinesterase is the termination of cholinergic neurotransmission, but the function of butyrylcholinesterase is not so clear [9, 10].

Cholinomimetics can be divided into two groups: direct cholinomimetics and indirect cholinomimetics. Direct cholinomimetics act directly on cholinergic receptors, whereas indirect cholinomimetics act as inhibitors of acetylcholinesterase [10].

Acetylcholinesterase inhibitors (AChEIs), which are classified as indirect cholinomimetics, have found use in the treatment of symptoms of Alzheimer's disease [10].

Carbamates, which are the most widely studied class of anticholinesterase agents, have attracted a great deal of interest among medicinal chemists and considerable research on them in relation to Alzheimer's disease has been accomplished. Rivastigmine, which is the dual AChE and BuChE inhibitor, is one of the most widely used anticholinesterase agents bearing carbamate group [1–10].

Many researchers have also investigated amides extensively due to the fact that new effective compounds can be obtained by the bioisosteric replacement of carbamate group with amide group. Some studies have confirmed that amide derivatives possess anticholinesterase activity [11–14].

Acetylcholine undergoes rapid hydrolysis in the gastrointestinal tract associated with its ester and quaternary ammonium salt functional groups and therefore acetylcholine cannot be administered orally [10]. In order to synthesize more metabolically stable cholinomimetic ligands, it is possible to replace the ester group with a series of five-membered rings like oxadiazoles, thiadiazoles, triazoles and tetrazoles [12]. Some researchers have studied thiadiazoles extensively and compounds bearing thiadiazole moiety have been reported to exhibit significant anticholinesterase activity [15–17].

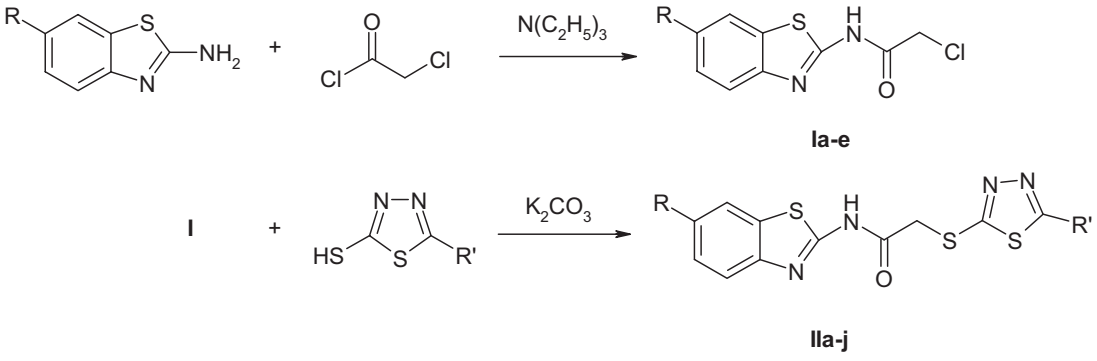
In this study, we described the synthesis of some amide derivatives bearing two functional moieties, namely benzothiazole and thiadiazole and focused on the potential anticholinesterase properties of these compounds. The prepared compounds were also investigated for their cytotoxic properties.

Results and discussion

Initially, 2-chloro-N-(benzothiazol-2-yl)acetamides (**Ia–e**) were obtained by reacting 2-aminobenzothiazoles with chloroacetyl chloride in the presence of triethylamine, which was responsible for the removal of hydrogen chloride from the reaction mixture.

The desired compounds (**IIa–j**) were synthesized via nucleophilic substitution reaction of 2-chloro-*N*-(benzothiazol-2-yl) acetamides with appropriate 1,3,4-thiadiazole-2-thioles in the presence of potassium carbonate. These reactions are summarized in Scheme 1 and some properties of the compounds are given in Table 1.

The structures of these compounds (**IIa–j**) were confirmed by ^{1}H-NMR, ^{13}C-NMR and FAB^{+}-MS spectral data and elemental analyses. In the ^{1}H-NMR spectra of the synthesized compounds, the signal due to the amide proton appears at 12–13 ppm as a broad peak. The signal due to the $-S-CH_2-$ protons gives rise to a singlet peak at 4.0–4.5 ppm. The benzothiazole protons of all derivatives are observed in the



Scheme 1. The synthetic protocol of the title compounds (**IIa–j**).

Table 1. Some properties of the synthesized compounds (**IIa–j**)

Compound	R	R′	Yield (%)	M.p. (°C)	Molecular formula	Molecular weight
IIa	H	CH_3	80	228–232	$C_{12}H_{10}N_4OS_3$	322
IIb	Cl	CH_3	85	258–261	$C_{12}H_9ClN_4OS_3$	356
IIc	CH_3	CH_3	82	252–255	$C_{13}H_{12}N_4OS_3$	336
IId	OCH_3	CH_3	84	244–248	$C_{13}H_{12}N_4O_2S_3$	352
IIe	OC_2H_5	CH_3	84	245–246	$C_{14}H_{14}N_4O_2S_3$	366
IIf	H	NH_2	85	250–253	$C_{11}H_9N_5OS_3$	323
IIg	Cl	NH_2	90	277–279	$C_{11}H_8ClN_5OS_3$	357
IIh	CH_3	NH_2	85	248–250	$C_{12}H_{11}N_5OS_3$	337
IIi	OCH_3	NH_2	88	260–262	$C_{12}H_{11}N_5O_2S_3$	353
IIj	OC_2H_5	NH_2	88	254–255	$C_{13}H_{13}N_5O_2S_3$	367

Table 2. The anticholinesterase activities of the compounds (**IIa–j**) as IC_{50} values (μg/mL)

Compound	AChE		BuChE	
	80 μg/mL (% inhibition)	IC_{50} μg/mL	80 μg/mL (% inhibition)	IC_{50} μg/mL
IIf	29,35 ± 0,28	>80	6,98 ± 4,09	>80
IIg	76,23 ± 1,67	70,33 ± 0,35	60,34 ± 5,19	72,50 ± 0,71
IIh	84,65 ± 2,97	66 ± 0,71	90,24 ± 1,17	48,75 ± 2,48
IIi	70,53 ± 2,60	72,75 ± 0,35	26,47 ± 2,50	>80
IIj	45,21 ± 2,08	>80	13,46 ± 2,87	>80
Eserine		$6,3 \times 10^{-3} \pm 0,03$		$4,7 \times 10^{-2} \pm 0,08$
Galanthamine		0,28 ± 0,04		7 ± 1,41

region 7.0–8.5 ppm. In the ^{1}H-NMR spectra of the compounds **IIf–j** the signal due to the amine protons appears at 7.35–7.38 ppm as a broad peak. Other aromatic and aliphatic protons were observed at expected regions.

In their ^{13}C-NMR spectra, the signal due to the amide carbon is observed at 165–170 ppm as a proof of the formation of amide moiety. The signal due to the $-S-CH_2-$ carbon appears at 36–38 ppm.

In the mass spectra of all compounds, the $[M+H]^+$ peak is observed. All compounds gave satisfactory elemental analysis.

The anticholinesterase activities of the compounds were determined by a modification of Ellman's spectrophotometric method (Table 2). All compounds showed less anticholinesterase potency than reference drugs.

5-Amino-1,3,4-thiadiazole derivatives (**IIf–j**) have anticholinesterase activity, whereas 5-methyl-1,3,4-thiadiazole derivatives (**IIa–e**) have no inhibitory effect on enzyme activity.

The results indicate that compound **IIh** is the most effective inhibitor of AChE and BuChE among these compounds. This outcome confirms that the amino group on thiadiazole ring and the methyl substituent on benzothiazole ring may have a considerable influence on anticholinesterase activity. When compared with reference drugs and other derivatives (**IIg–IIj**), compound **IIf** exhibited the lowest anticholinesterase activity.

The compounds were also evaluated for their cytotoxic properties using MTT assay (Table 3). The biological study indicated that compound **IIf** possessed the highest cytotoxicity, whereas compound **IIi** exhibited the lowest cytotoxicity *in vitro* against NIH/3T3 cells among the title compounds.

Table 3. *In vitro* cytotoxicity of the compounds (**IIa–j**)

Compound	IC_{50} (μg/mL)[a]
IIa	60 ± 10
IIb	76,7 ± 5,8
IIc	45,7 ± 14,0
IId	80 ± 10
IIe	66,6 ± 15,27
IIf	33,3 ± 5,8
IIg	63,3 ± 5,7
IIh	70 ± 8,7
IIi	90 ± 10
IIj	70 ± 10

[a] Cytotoxicity of compounds to mouse fibroblast (NIH/3T3) cell line. Incubation for 24 h. IC_{50} is the drug concentration required to inhibit 50% of the cell growth. The values represent mean ± standard deviation of triplicate determinations.

Conclusion

In conclusion, we synthesized some amide derivatives and evaluated their anticholinesterase activities and cytotoxic properties. The results indicate that compound **IIh** is the most potent inhibitor of AChE and BuChE among these compounds. Furthermore, the cytotoxicity of compound **IIh** is lower than its effective dose (66 ± 0.71 μg/mL). This outcome indicates that the amino group on thiadiazole ring and the methyl substituent on benzothiazole ring have a considerable influence on the ability to inhibit cholinesterases.

Experimental

Chemistry

All reagents were purchased from commercial suppliers and were used without further purification. Melting points (m.p.) were determined on a Electrothermal 9100 melting point apparatus (Weiss-Gallenkamp, Loughborough, UK) and are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Bruker 400 MHz spectrometer (Bruker, Billerica, MA, USA). Carbon nuclear magnetic resonance (^{13}C-NMR) spectra were recorded on a Bruker 100 MHz spectrometer (Bruker, Billerica, MA, USA). Chemical shifts were expressed in parts per million (ppm) and tetramethylsilane was used as an internal standard. Mass spectra were recorded on a VG Quattro Mass spectrometer (Agilent, Minnesota, USA). Elemental analyses were performed on a Perkin Elmer EAL 240 elemental analyser (Perkin-Elmer, Norwalk, CT, USA).

General procedure for the synthesis of the compounds

*N-(Benzothiazol-2-yl)-2-chloroacetamides (**Ia–e**)*

Chloroacetyl chloride (0,1 mol) was added dropwise with stirring to a mixture of 2-aminobenzothiazole derivatives (0,1 mol) and triethylamine (0,1 mol) in toluene (50 mL) at 0–5°C. The solvent was evaporated under reduced pressure. The residue was washed with water to remove triethylamine hydrochloride and crystallized from ethanol [18].

*N-(Benzothiazol-2-yl)-2-[(5-amino/methyl-1,3,4-thiadiazol-2-yl)thio]acetamide derivatives (**IIa–j**)*

A mixture of *N*-(benzothiazol-2-yl)-2-chloroacetamide derivatives (**Ia–e**) (2 mmol) and 5-amino/methyl-1,3,4-thiadiazole-2-thiole (2 mmol) in acetone (10 mL) was stirred at room temperature for 8 hours in the presence of potassium carbonate (2 mmol) and filtered. The residue was washed with water and crystallized from ethanol.

*2-(5-Methyl-1,3,4-thiadiazol-2-yl)thio-N-(benzothiazol-2-yl)-acetamide (**IIa**)*

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.67 (3H, s), 4.44 (2H, s), 7.32 (1H, t, J = 7.7 Hz), 7.45 (1H, t, J = 7.7 Hz), 7.77 (1H, d, J = 7.7 Hz), 7.98 (1H, d, J = 7.7 Hz), 12.75 (1H, br).

^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 15.17 (CH_3), 36.80 (CH_2), 120.66 (CH), 121.74 (CH), 123.68 (CH), 126.18 (CH), 131.42 (C), 148.47 (C), 157.65 (C), 163.80 (C), 165.91 (C), 166.74 (C).

For $C_{12}H_{10}N_4OS_3$ calculated: C, 44.70; H, 3.13; N, 17.38; found: C, 44.72; H, 3.12; N, 17.36.

MS (FAB) $[M+1]^+$: m/z 323

*2-(5-Methyl-1,3,4-thiadiazol-2-yl)thio-N-(6-chlorobenzothiazol-2-yl)acetamide (**IIb**)*

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.67 (3H, s), 4.43 (2H, s), 7.47 (1H, dd, J = 8.6, 2.1 Hz), 7.77 (1H, t, J = 8.6 Hz), 8.14 (1H, d, J = 2.1 Hz), 12.83 (1H, br).

^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 15.17 (CH_3), 36.72 (CH_2), 121.49 (CH), 121.88 (CH), 126.55 (CH), 127.74 (C), 133.12 (C), 147.37 (C), 158.52 (C), 163.74 (C), 165.96 (C), 166.99 (C).

For $C_{12}H_9ClN_4OS_3$ calculated: C, 40.39; H, 2.54; N, 15.70; found: C, 40.40; H, 2.56; N, 15.69.

MS (FAB) $[M\pm1]^+$: m/z 357

*2-(5-Methyl-1,3,4-thiadiazol-2-yl)thio-N-(6-methylbenzothiazol-2-yl)acetamide (**IIc**)*

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.49 (3H, s), 2.71 (3H, s), 4.48 (2H, s), 7.51 (1H, dd, J = 8.6, 2.1 Hz), 7.71 (1H, t, J = 8.6 Hz), 8.20 (1H, d, J = 2.1 Hz), 12.89 (1H, br).

^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 15.11 (CH_3), 20.99 (CH_3), 36.60 (CH_2), 121.89 (CH), 122.11 (CH), 127.15 (CH), 127.99 (C), 134.01 (C), 148.02 (C), 159.02 (C), 164.02 (C), 166.52 (C), 167.52 (C).

For $C_{13}H_{12}N_4OS_3$ calculated: C, 46.41; H, 3.59; N, 16.65; found: C, 46.40; H, 3.60; N, 16.64.

MS (FAB) $[M\pm1]^+$: m/z 337

*2-(5-Methyl-1,3,4-thiadiazol-2-yl)thio-N-(6-methoxybenzothiazol-2-yl)acetamide (**IId**) [19]*

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.70 (3H, s), 3.83 (3H, s), 4.49 (2H, s), 7.00 (1H, dd, J = 8.7, 1.8 Hz), 7.62 (1H, d, J = 1.8 Hz), 7.69 (1H, d, J = 8.7 Hz), 12.53 (1H, br).

^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 15.16 (CH_3), 36.80 (CH_2), 55.99 (CH_3), 105.85 (CH), 114.21 (CH), 122.45 (CH), 133.65 (C), 141.20 (C), 149.95 (C), 156.29 (C), 157.21 (C), 167.42 (C), 169.99 (C).

For $C_{13}H_{12}N_4O_2S_3$ calculated: C, 44.30; H, 3.43; N, 15.90; found: C, 44.29; H, 3.42; N, 15.91.

MS (FAB) $[M\pm1]^+$: m/z 353

*2-(5-Methyl-1,3,4-thiadiazol-2-yl)thio-N-(6-ethoxybenzothiazol-2-yl)acetamide (**IIe**)*

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.36 (3H, t, J = 7.1 Hz), 2.72 (3H, s), 4.06 (2H, q, J = 7.1 Hz), 4.51 (2H, s), 7.05 (1H, dd, J = 8.8, 1.8 Hz), 7.63 (1H, d, J = 1.8 Hz), 7.72 (1H, d, J = 8.8 Hz), 12.60 (1H, br).

^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 14.60 (CH_3), 15.18 (CH_3), 37.45 (CH_2), 63.66 (CH_2), 105.95 (CH), 114.36 (CH), 122.25 (CH), 134.64 (C), 143.11 (C), 147.12 (C), 155.45 (C), 155.60 (C), 166.75 (C), 169.90 (C).

For $C_{14}H_{14}N_4O_2S_3$ calculated: C, 45.88; H, 3.85; N, 15.29; found: C, 45.84; H, 3.88; N, 15.30.

MS (FAB) $[M+1]^+$: m/z 367

*2-(5-Amino-1,3,4-thiadiazol-2-yl)thio-N-(benzothiazol-2-yl)acetamide (**IIf**)*

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 4.21 (2H, s), 7.33 (1H, t, J = 7.8 Hz), 7.37 (2H, br), 7.48 (1H, t, J = 7.8 Hz), 7.83 (1H, d, J = 7.8 Hz), 8.02 (1H, d, J = 7.8 Hz), 12.71 (1H, br).

^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 36.80 (CH_2), 105.48 (CH), 114.90 (CH), 122.14 (CH), 131.96 (CH), 141.92 (C), 147.15 (C), 154.61 (C), 155.82 (C), 167.02 (C), 169.85 (C).

For $C_{11}H_9N_5OS_3$ calculated: C, 40.85; H, 2.80; N, 21.65; found: C, 40.88; H, 2.84; N, 21.63.

MS (FAB) $[M\pm1]^+$: m/z 324

*2-(5-Amino-1,3,4-thiadiazol-2-yl)thio-N-(6-chlorobenzothiazol-2-yl)acetamide (**IIg**)*

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 4.21 (2H, s), 7.26 (1H, dd, J = 8.5, 2.0 Hz), 7.37 (2H, br), 7.60 (1H, t, J = 8.5 Hz), 7.75 (1H, d, J = 2.0 Hz), 12.62 (1H, br).

^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 36.82 (CH_2), 122.09 (CH), 122.90 (CH), 126.75 (CH), 127.82 (C), 133.42 (C), 148.07 (C), 158.90 (C), 163.68 (C), 166.06 (C), 167.05 (C).

For $C_{11}H_8ClN_5OS_3$ calculated: C, 36.92; H, 2.25; N, 19.57; found: C, 36.90; H, 2.28; N, 19.60.

MS (FAB) $[M\pm1]^+$: m/z 358

*2-(5-Amino-1,3,4-thiadiazol-2-yl)thio-N-(6-methylbenzothiazol-2-yl)acetamide (**IIh**)*

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 2.45 (3H, s), 4.16 (2H, s), 7.09 (1H, dd, J = 8.7, 1.9 Hz), 7.38 (2H, br), 7.61 (1H, d, J = 1.9 Hz), 7.69 (1H, d, J = 8.7 Hz), 12.51 (1H, br).

^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 21.10 (CH_3), 37.11 (CH_2), 105.48 (CH), 115.21 (CH), 122.01 (CH), 131.99 (C), 141.95 (C), 147.12 (C), 156.05 (C), 156.99 (C), 167.12 (C), 169.12 (C).

For $C_{12}H_{11}N_5OS_3$ calculated: C, 42.71; H, 3.29; N, 20.75; found: C, 42.70; H, 3.30; N, 20.72.

MS (FAB) $[M\pm1]^+$: m/z 338

*2-(5-Amino-1,3,4-thiadiazol-2-yl)thio-N-(6-methoxybenzothiazol-2-yl)acetamide (**IIi**) [19]*

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 3.81 (3H, s), 4.14 (2H, s), 7.04 (1H, dd, J = 8.8, 1.9 Hz), 7.35 (2H, br), 7.58 (1H, d, J = 1.9 Hz), 7.65 (1H, d, J = 8.8 Hz), 12.50 (1H, br).

^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 37.40 (CH_2), 55.59 (CH_3), 104.68 (CH), 115.00 (CH), 121.24 (CH), 132.76 (C), 142.52 (C), 148.75 (C), 155.59 (C), 156.20 (C), 166.95 (C), 170.09 (C).

For $C_{12}H_{11}N_5O_2S_3$ calculated: C, 40.78; H, 3.14; N, 19.81; found: C, 40.75; H, 3.18; N, 19.79.

MS (FAB) $[M+1]^+$: *m/z* 354

*2-(5-Amino-1,3,4-thiadiazol-2-yl)thio-N-(6-ethoxybenzothiazol-2-yl)acetamide (**IIj**)*

^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.34 (3H, t, $J = 7.0$ Hz), 4.05 (2H, q, $J = 7.0$ Hz), 4.14 (2H, s), 7.02 (1H, dd, $J = 8.8, 2.6$ Hz), 7.36 (2H, br), 7.54 (1H, d, $J = 2.6$ Hz), 7.64 (1H, d, $J = 8.8$ Hz), 12.48 (1H, br).

^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 14.65 (CH_3), 37.41 (CH_2), 63.56 (CH_2), 105.30 (CH), 115.33 (CH), 121.23 (CH), 132.74 (C), 142.44 (C), 148.73 (C), 155.43 (C), 155.55 (C), 166.93 (C), 170.10 (C).

For $C_{13}H_{13}N_5O_2S_3$ calculated: C, 42.49; H, 3.57; N, 19.06; found: C, 42.48; H, 3.56; N, 19.05.

MS (FAB) $[M+1]^+$: *m/z* 368

Pharmacology

AChE and BuChE inhibitory activity

AChE and BuChE inhibitory activity was determined by Ellman's method with minor modifications [20]. Compounds **IIa–j** were dissolved in DMSO and were tested at final concentration range 5–80 μg/mL. 20 μL of enzyme (AChE or BuChE, 1 u/mL) and 10 μL sample were added to 2.4 mL buffer, the mixture was incubated at 37°C for 15 min. After the 15 min incubation, 50 μL of 0.01 M DTNB and 20 μL of 75 mM ATCI or 10 mM BTCI were added, and the final mixture was incubated at room temperature for 30 min. A control mixture (blank) was prepared using 10 μL of DMSO instead of the test sample, with all other procedures similar to those used in the case of the sample mixture. Absorbances were measured at 412 nm and 37°C using polystyrol cuvets with specrophotometer (Shimadzu, UV-1700). Experiment was done in triplicate. Data are expressed as mean ± standard deviation (SD).

The inhibition (percent) of AChE and BuChE was calculated using the following equation.

$$\mathrm{I}(\%) = 100\text{-}(\mathrm{OD}_{\mathrm{sample}}/\mathrm{OD}_{\mathrm{control}}) \times 100$$

Toxicity

The level of cellular MTT (Sigma) reduction was quantified as previously described in the literature with small modifications [21, 22].

Cell culture and drug treatment

NIH/3T3 cells were obtained from the American Type Culture Collection (ATCC, USA). The cells were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Life Technologies, UK), 100 IU/mL penicillin (Gibco, Paisley, Scotland) and 100 mg/mL streptomycin (Gibco) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Exponentially growing cells were plated at 2×10^4 cells/mL into 96-well microtiter tissue culture plates (Nunc, Denmark) and incubated for 24 h before the addition of the drugs (the optimum cell number for cytotoxicity assays was determined in preliminary experiments). Stock solutions of compounds were prepared in dimethyl sulphoxide (DMSO; Sigma-Aldrich, Poole, UK) and further dilutions were made with fresh culture medium (the concentration of DMSO in the final culture medium was <0.1% which had no effect on the cell viability).

MTT assay for cytotoxicity of the compounds

It is widely used as a measure of cytotoxicity. After 24 h of preincubation, the tested compounds were added to give final concentration in the range 0.5–500 μg/mL and the cells were incubated for 24 h. At the end of this period, MTT was added to a final concentration of 0.5 mg/mL and the cells were incubated for 4 h at 37°C. After the medium was removed, the formazan crystals formed by MTT metabolism were solubilized by addition of 200 μL DMSO to each well and absorbance was read at 540 nm with a microtitre plate spectrophotometer (Bio-Tek plate reader). Every concentration was repeated in three wells and IC_{50} values were defined as the drug concentrations that reduced absorbance to 50% of control values.

The authors have declared no conflict of interest.

References

[1] A. Martinez, A. Castro, *Expert Opin. Investig. Drugs* **2006**, *15*, 1–12.

[2] E. Giacobini, *Neurochem. Res.* **2003**, *28*, 515–522.

[3] Z.-X. Shen, *Med. Hypotheses* **2004**, *63*, 298–307.

[4] J. Grutzendler, J. C. Morris, *Drugs* **2001**, *61*, 41–52.

[5] G. Pepeu, M. G. Giovannini, *Curr. Alzheimer Res.* **2009**, *6*, 86–96.

[6] J. B. Standridge, *Clin. Ther.* **2004**, *26*, 615–630.

[7] P. Johannsen, *CNS Drugs* **2004**, *18*, 757–768.

[8] G. T. Grossberg, *Curr. Ther. Res. Clin. Exp.* **2003**, *64*, 216–235.

[9] D. G. Wilkinson, P. T. Francis, E. Schwam, J. Payne-Parrish, *Drugs Aging* **2004**, *21*, 453–478.

[10] T. L. Lemke, D. A. Williams, in: *Foye's Principles of Medicinal Chemistry*, Lippincott Williams&Wilkins, Baltimore and Philadelphia, USA **2008**, chapter 12.

[11] G. A. Patani, E. J. LaVoie, *Chem. Rev.* **1996**, *96*, 3147–3176.

[12] P. Ciapetti, B. Giethlen, in Molecular Variations Based on Isosteric Replacements. in C. M. Wermuth, *The Practice of Medicinal Chemistry*, Academic Press Burlington, USA **2008**, p. 290–342.

[13] S. Darvesh, R. S. McDonald, A. Penwell, S. Conrad, K. V. Darvesh, D. Mataija, G. Gomez, A. Caines, R. Walsh, E. Martin, *Bioorg. Med. Chem.* **2005**, *13*, 211–222.

[14] S. Darvesh, R. S. McDonald, K. V. Darvesh, D. Mataija, S. Conrad, G. Gomez, R. Walsh, E. Martin, *Bioorg. Med. Chem.* **2007**, *15*, 6367–6378.

[15] A. K. Sengupta, M. Garg, *J. Indian Chem. Soc.* **1980**, 1241–1243.

[16] H. K. Misra, *Arch. Pharm.* **1983**, *316*, 487–493.

[17] A. K. Sen Gupta, H. K. Misra, *J. Pharm. Sci.* **1980**, *69*, 1313–1317.

[18] P. N. Bhargava, P. Ram, *Bull. Chem. Soc. Japan* **1965**, *38*, 339–341.

[19] D. S. Goldfarb, US 20090163545 A1. **2009**.

[20] G. L. Ellman, K. D. Courtney, V. J. Anders, R. M. Featherstone, *Biochem. Pharmacol.* **1961**, *7*, 88–95.

[21] T. Mossmann, *J. Immunol. Methods* **1983**, *65*, 55–63.

[22] K. Keiser, C. C. Johnson, D. A. Tipton, *J. Endod.* **2000**, *26*, 288–291.

