Full Paper

Synthesis and Biological Evaluation of a Series of Dithiocarbamates as New Cholinesterase Inhibitors

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In the present paper, a novel series of dithiocarbamates was synthesized via the treatment of 4-(trifluoromethyl)benzyl chloride with appropriate sodium salts of *N*,*N*-disubstituted dithiocarbamic acids. The chemical structures of the compounds were elucidated by ^{1}H NMR, mass spectral data, and elemental analyses. Each derivative was evaluated for its ability to inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) using a modification of Ellman's spectrophotometric method. The most potent AChE inhibitor was found as compound **2g** ($IC_{50} = 0.53 \pm 0.001$ μM) followed by compounds **2f** ($IC_{50} = 0.74 \pm 0.001$ μM) and **2j** ($IC_{50} = 0.89 \pm 0.002$ μM) when compared with donepezil ($IC_{50} = 0.048 \pm 0.001$ μM). Compounds **2f** and **2g** were more effective than donepezil ($IC_{50} = 7.88 \pm 0.52$ μM) on BuChE inhibition. Compounds **2f** and **2g** exhibited the inhibitory effect on BuChE with IC_{50} values of 1.39 ± 0.041 and 3.64 ± 0.072 μM, respectively.

Keywords: Acetylcholinesterase / Anticholinesterase / Butyrylcholinesterase / Dithiocarbamate

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Introduction

Inhibition of disease-associated enzymes by small molecule drugs is a promising approach for pharmacologic intervention in human disease. The catalytic activity of specific enzymes is often critical to the pathophysiology of disease, such that inhibition of catalysis is disease modifying. The binding pockets for natural ligands of enzymes are often uniquely well-suited for interactions with small molecule drugs. Thus, the very nature of the chemistry of enzyme catalysis makes these proteins amenable to inhibition by small molecular weight, drug-like molecules [1, 2].

Cholinesterases (ChEs) remain a major focus of pharmaceutical research for the treatment of some of the symptoms of Alzheimer's disease (AD) owing to the fundamental roles of ChEs in normal brain structure and function and in the initiation and development of AD. Two ChEs are present in humans: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Both ChEs are present in cholinergic synapses in the central nervous system (CNS), in the parasympathic synapses in the periphery, and in the neuromuscular junction. Whereas AChE is selective for ACh hydrolysis, BuChE hydrolyses acetylcholine and other choline esters as a non-specific cholinesterase [1–9].

Carbamates are the most widely studied class of anticholinesterase agents and considerable research on them in relation to Alzheimer's disease has been accomplished. Rivastigmine, a dual AChE and BuChE inhibitor, is one of the most widely used anticholinesterase agents bearing carbamate group, which resembles the ester linkage of acetylcholine [3–10].

Dithiocarbamates have attracted a great deal of interest in medicinal chemistry due to the fact that new effective compounds can be obtained by the bioisosteric replacement of carbamate moiety with dithiocarbamate moiety. They are also important pharmacophores due to their lipophilicity, which is crucial for the delivery of CNS drugs to their site of action through the blood–brain barrier [11–18].

On the basis of these findings and in the continuation of our ongoing research program in the field of synthesis and biological evaluation of heterocyclic compounds as cholinesterase inhibitors [19, 20], herein we report the synthesis and biological evaluation of some dithiocarbamate derivatives as new anticholinesterase agents.

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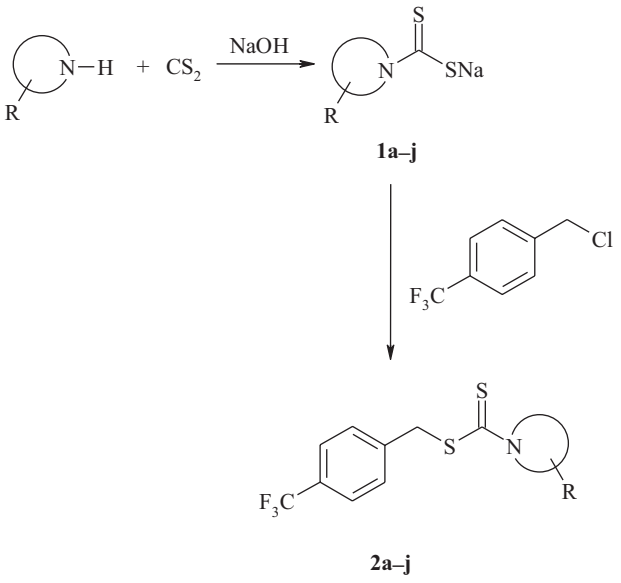
Results and discussion

The synthesis of dithiocarbamate derivatives **2a–j** was carried out according to the steps shown in Scheme 1. Sodium salts of *N*,*N*-disubstituted dithiocarbamic acids were obtained by the reaction of secondary amine with carbon disulfide in the presence of sodium hydroxide.

The reaction of 4-(trifluoromethyl)benzyl chloride with sodium salts of *N*,*N*-disubstituted dithiocarbamic acids **1a–j** afforded dithiocarbamate derivatives **2a–j**. Some properties of the compounds are given in Table 1.

The anticholinesterase effects of the compounds **2a–j** on AChE and BuChE were determined by a modification of Ellman's spectrophotometric method (Table 2). Donepezil, a selective AChE inhibitor, was used as the reference drug [9].

The enzymatic assay indicated that piperazine derivatives were more effective than other derivatives on AChE inhibition. Among piperazine derivatives, compound **2g** can be identified as the most promising anticholinesterase agent due to its inhibitory effect on AChE with an IC_{50} value of 0.53 ± 0.001 μM when compared with donepezil ($IC_{50} = 0.048 \pm 0.001$ μM). Compounds **2f** and **2j** exhibited AChE



Scheme 1. The synthesis of the compounds **2a–j**.

Table 1. Some properties of the compounds **2a–j**.

Compound	Ring	R	Yield (%)	m.p. (°C)	Molecular formula	Molecular weight
2a	Thiomorpholinyl	H	87	68	$C_{13}H_{14}F_3NS_3$	337.45
2b	Morpholinyl	H	85	72	$C_{13}H_{14}F_3NOS_2$	321.38
2c	Pyrrolidinyl	H	76	73	$C_{13}H_{14}F_3NS_2$	305.38
2d	Piperidinyl	H	79	52	$C_{14}H_{16}F_3NS_2$	319.41
2e	Piperidinyl	4-Methyl	79	69	$C_{15}H_{18}F_3NS_2$	333.44
2f	Piperazinyl	4-Methyl	80	75	$C_{14}H_{17}F_3N_2S_2$	334.42
2g	Piperazinyl	4-Ethyl	78	73.5	$C_{15}H_{19}F_3N_2S_2$	348.45
2h	Piperazinyl	4-Phenyl	82	113	$C_{19}H_{19}F_3N_2S_2$	396.49
2i	Piperazinyl	4-(4-Methoxyphenyl)	80	87	$C_{20}H_{21}F_3N_2OS_2$	426.52
2j	Piperazinyl	4-(2-Pyrimidinyl)	83	140	$C_{17}H_{17}F_3N_4S_2$	398.47

Table 2. AChE/BuChE % inhibition of the compounds **2a–j** and IC_{50} values.

	AChE inhibition (%)				BuChE inhibition (%)		
Compound	**100 (μM)**	**1 (μM)**	**0.01 (μM)**	**IC_{50} (μM)**	**100 (μM)**	**1 (μM)**	**IC_{50} (μM)**
2a	30.59 ± 3.21	NC	NC	>100	32.23 ± 4.16	NC	>100
2b	30.51 ± 5.16	NC	NC	>100	24.57 ± 3.41	NC	>100
2c	34.38 ± 5.15	NC	NC	>100	21.93 ± 4.38	NC	>100
2d	39.81 ± 4.37	NC	NC	>100	36.09 ± 5.38	NC	>100
2e	24.80 ± 3.64	NC	NC	>100	28.82 ± 3.27	NC	>100
2f	85.92 ± 4.71	58.26 ± 2.73	14.54 ± 1.12	0.74 ± 0.001	74.63 ± 3.67	38.71 ± 2.59	1.39 ± 0.041
2g	87.30 ± 4.42	62.75 ± 2.56	36.27 ± 3.83	0.53 ± 0.001	72.24 ± 4.50	23.64 ± 3.93	3.64 ± 0.072
2h	37.51 ± 4.46	NC	NC	>100	29.34 ± 1.73	NC	>100
2i	25.84 ± 5.37	NC	NC	>100	29.47 ± 2.09	NC	>100
2j	79.73 ± 6.28	56.61 ± 3.19	15.37 ± 1.23	0.89 ± 0.002	14.35 ± 1.43	NC	>100
Donepezil	97.23 ± 4.27	81.69 ± 3.36	36.42 ± 5.41	0.048 ± 0.001	70.62 ± 4.26	38.29 ± 2.81	7.88 ± 0.52

NC, not calculated.

inhibitory activity with IC_{50} values of 0.74 ± 0.001 and 0.89 ± 0.002 μM, respectively. On the other hand, compounds **2h** and **2i** bearing a piperazine moiety showed weak inhibition on AChE ($IC_{50} > 100$ μM). This outcome confirms that the substituents at the 4th position of the piperazine ring may have a considerable influence on AChE inhibition.

Compounds **2f** and **2g** were more effective than donepezil on BuChE inhibition. Compound **2f** exhibited the highest inhibitory effect on BuChE with an IC_{50} value of 1.39 ± 0.041 μM when compared with donepezil ($IC_{50} = 7.88 \pm 0.52$ μM). Compound **2g** exhibited BuChE inhibitory activity with an IC_{50} value of 3.64 ± 0.072 μM. Although compound **2j** bearing a pyrimidine moiety at the 4th position of the piperazine ring showed AChE inhibitory activity with an IC_{50} value of 0.89 ± 0.002 μM, it showed weak inhibition on BuChE ($IC_{50} > 100$ μM). Other derivatives showed weak inhibition on BuChE ($IC_{50} > 100$ μM). It is apparent that there is a positive correlation between BuChE inhibitory activity and the 4-alkylpiperazine moiety.

The kinetics of this new class of AChE inhibitors were studied in detail using the most active compound **2g**. The nature of AChE inhibition, caused by this compound, was investigated by the graphical analysis of steady-state inhibition data (Fig. 1). Reciprocal plots (Lineweaver–Burk plots) described compound **2g** as a mixed type inhibitor, due to different intercepts on both the *y*- and *x*-axes. The values of K_m and V_{max} were calculated by nonlinear regression according to Heng et al. [21]. For compound **2g**, the K_m and V_{max} values were 3.18 and 3.23, respectively.

In order to gain more insight into whether the ligands inhibit AChE, docking study was implemented using the X-ray structure of *Torpedo californica* acetylcholinesterase (*Tc*AChE; PDB ID: 3I6Z), which was previously used by Razavi et al. [22] for the development of some AChE inhibitors. As the structure of *Tc*AChE is similar to *Electrophorus electricus* AChE (electric eel AChE), it was used as the receptor model for docking studies [22]. Novel compound **2g** was docked into the binding site of the X-ray structure of *Tc*AChE. Low energy docked coordinates was selected to analyze the most likely interaction with the receptor, based on the thiocarbamate pharmacophore showing the best superposition with that of the X-ray coordinates of *N*-saccharinohexyl-galanthamine. Amino acids in the active site of *Tc*AchE and the interaction of the best docking pose of compound **2g** are shown in Fig. 2. In the molecular docking study, it was observed that the molecule was compatible with the active site thanks to the residues Phe330, Phe331, Tyr334 at the bottom and Trp279 at the entrance of the binding pocket. It was shown that compound **2g** was settled down with the formation of π–π interaction between phenyl ring of 4-(trifluoromethyl)benzyl moiety and phenyl ring of the Phe331 residue at the bottom of the active site. The formation of an interaction between piperazine moiety and phenyl ring of Trp279 residue was also observed at the entrance of the active site of the enzyme (Fig. 2A). These interactions stabilize the ligand in the binding pocket of the enzyme (Fig. 2B), but no hydrogen bonding was observed in the active site.

Conclusion

In the present work, we described the synthesis of a series of dithiocarbamate derivatives, which were evaluated for their anticholinesterase effects on AChE and BuChE.

Piperazine derivatives were more effective than other derivatives on AChE inhibition. The most potent AChE inhibitor was found as compound **2g** ($IC_{50} = 0.53 \pm 0.001$ μM) followed by compounds **2f** ($IC_{50} = 0.74 \pm 0.001$ μM) and **2j** ($IC_{50} = 0.89 \pm 0.002$ μM) when compared with donepezil ($IC_{50} = 0.048 \pm 0.001$ μM). Although compounds **2h** and **2i** carry a piperazine moiety, they showed weak inhibition on AChE ($IC_{50} > 100$ μM). The substituents at

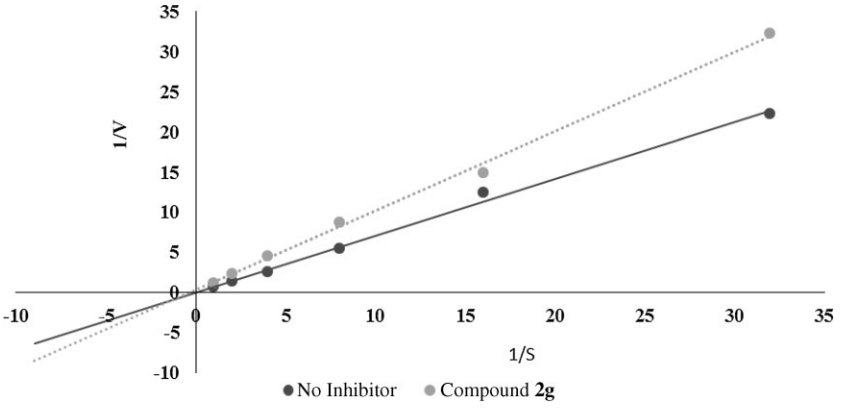


Figure 1. Lineweaver–Burk plots for compound **2g** ($IC_{50} = 0.53$ μM). Substrate (ATCI) concentrations used: 0.5, 0.25, 0.125, 0.0625, 0.03125 μM. 1/*V*: 1/velocity of reaction [1/(absorbance/1 min)], 1/*S*: 1/substrate concentration (1/μM ATCI).

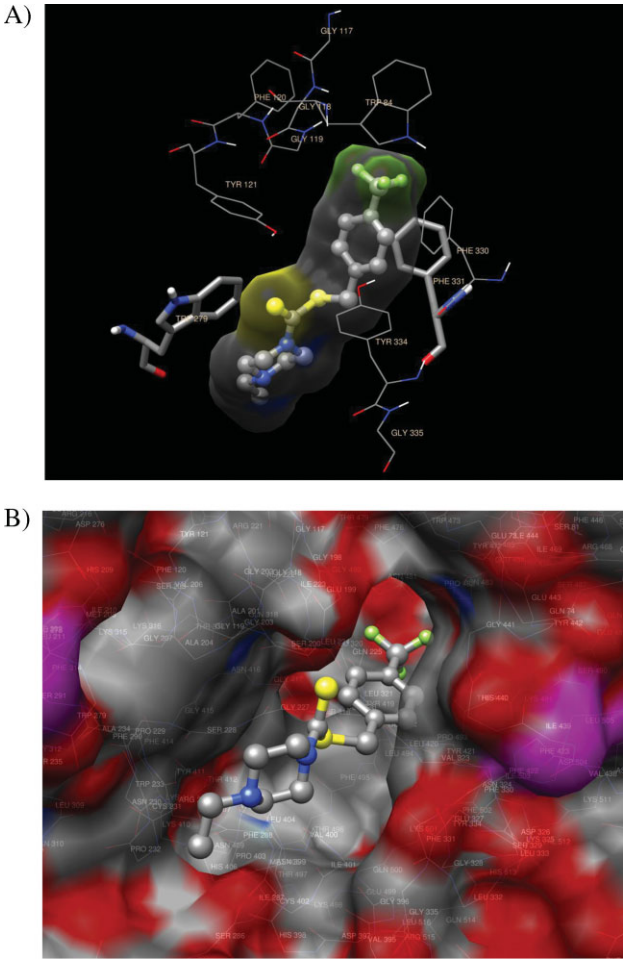


Figure 2. View of the active site of *Tc*AChE (PDB ID: 3I6Z) in complex with compound **2g** constructed by UCSF Chimera package [29]. (A) Amino acid residues in the active site; (B) ligand binding pocket of the enzyme.

the 4th position of the piperazine ring also have a considerable influence on AChE inhibition.

Compounds **2f** and **2g** were found to be more effective than donepezil ($IC_{50} = 7.88 \pm 0.52$ μM) on BuChE inhibition. Compounds **2f** and **2g** exhibited the inhibitory effect on BuChE with IC_{50} values of 1.39 ± 0.041 and 3.64 ± 0.072 μM, respectively. It is clear that there is a positive correlation between BuChE inhibitory activity and the 4-alkylpiperazine moiety.

The kinetics of this new class of AChE inhibitors were studied in detail using the most active compound **2g**. According to the results, compound **2g** was described as a mixed type inhibitor. Molecular docking study of compound **2g** was also carried out. Based on the molecular docking study, compound **2g** might be involved in the interaction with *Tc*AChE (PDB ID: 3I6Z) and considered as an AChE inhibitor.

Experimental

Chemistry

All reagents were purchased from commercial suppliers and 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 500 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

*Sodium salts of N,N-disubstituted dithiocarbamic acids (**1a–j**)*

Sodium hydroxide (10 mmol) was dissolved in ethanol (80 mL) with constant stirring. After addition of the secondary amine (10 mmol) the mixture was cooled in an ice bath and carbon disulfide (100 mmol) was added dropwise with stirring. The reaction mixture was stirred for 1 h at room temperature. The products were afforded by filtration and washed with diethyl ether [18].

*Dithiocarbamate derivatives (**2a–j**)*

A mixture of 4-(trifluoromethyl)benzyl chloride (0.01 mol) and appropriate sodium salts of *N*,*N*-disubstituted dithiocarbamic acids (0.01 mol) was treated in acetone (15 mL) at room temperature for 3 h. The solvent was evaporated, the resulting solid was washed with water, and recrystallized from ethanol.

*4-(Trifluoromethyl)benzyl thiomorpholine-4-carbodithioate (**2a**)*

^{1}H NMR (δ ppm) (DMSO-d_6): 2.70 (4H, t, C_3 and C_5 protons of thiomorpholine), 4.22 and 4.53 (4H, two brs, C_2 and C_6 protons of thiomorpholine), 4.68 (2H, s, $COCH_2$), 7.62 and 7.68 (4H, two d (J = 8 and 8 Hz), 1,4-disubstituted phenyl protons).

For $C_{13}H_{14}F_3NS_3$, calcd.: C, 46.27; H, 4.18; N, 4.15; Found: C, 46.28; H, 4.15; N, 4.12.

MS (ES): $[M+1]^+$: 338.

*4-(Trifluoromethyl)benzyl morpholine-4-carbodithioate (**2b**)*

^{1}H NMR (δ ppm) (DMSO-d_6): 3.67 (4H, s, C_3 and C_5 protons of morpholine), 3.92 and 4.24 (4H, two brs, C_2 and C_6 protons of morpholine), 4.69 (2H, s, $COCH_2$), 7.62 and 7.67 (4H, two d (J = 8.5 and 8.5 Hz), 1,4-disubstituted phenyl protons).

For $C_{13}H_{14}F_3NOS_2$, calcd.: C, 48.58; H, 4.39; N, 4.36; Found: C, 48.60; H, 4.37; N, 4.33.

MS (ES): $[M+1]^+$: 322.

*4-(Trifluoromethyl)benzyl pyrrolidine-1-carbodithioate (**2c**)*

^{1}H NMR (δ ppm) (DMSO-d_6): 1.90 and 2.01 (4H, two p, C_3 and C_4 protons of pyrrolidine), 3.61 and 3.79 (4H, two t, C_2 and C_5 protons

of pyrrolidine), 4.68 (2H, s, $COCH_2$), 7.62 and 7.67 (4H, two d (J = 7 and 8 Hz), 1,4-disubstituted phenyl protons).

For $C_{13}H_{14}F_3NS_2$, calcd.: C, 51.13; H, 4.62; N, 4.59; Found: C, 51.15; H, 4.60; N, 4.58.

MS (ES): $[M+1]^+$: 306.

*4-(Trifluoromethyl)benzyl piperidine-1-carbodithioate (**2d**)*

^{1}H NMR (δ ppm) (DMSO-d_6): 1.53–1.61 (4H, m, C_3 and C_5 protons of piperidine), 1.62–1.68 (2H, m, C_4 protons of piperidine), 3.88 and 4.23 (4H, two s, C_2 and C_6 protons of piperidine), 4.66 (2H, s, $COCH_2$), 7.61 and 7.67 (4H, two d (J = 8 and 8 Hz), 1,4-disubstituted phenyl protons).

For $C_{14}H_{16}F_3NS_2$, calcd.: C, 52.64; H, 5.05; N, 4.39; Found: C, 52.63; H, 5.07; N, 4.37.

MS (ES): $[M+1]^+$: 320.

*4-(Trifluoromethyl)benzyl 4-methylpiperidine-1-carbodithioate (**2e**)*

^{1}H NMR (δ ppm) (DMSO-d_6): 0.90 (3H, d (J = 6 Hz), CH_3), 1.02–1.15 (2H, m, piperidine protons), 1.68–1.77 (3H, m, piperidine protons), 3.15–3.35 (2H, m, piperidine protons), 4.38–4.49 (1H, m, piperidine proton), 4.66 (2H, d (J = 10 Hz), $COCH_2$), 5.22–5.33 (1H, m, piperidine proton), 7.60 and 7.66 (4H, two d (J = 8 and 8.5 Hz), 1,4-disubstituted phenyl protons).

For $C_{15}H_{18}F_3NS_2$, calcd.: C, 54.03; H, 5.44; N, 4.20; Found: C, 54.05; H, 5.43; N, 4.19.

MS (ES): $[M+1]^+$: 334.

*4-(Trifluoromethyl)benzyl 4-methylpiperazine-1-carbodithioate (**2f**)*

^{1}H NMR (δ ppm) (DMSO-d_6): 2.19 (3H, s, CH_3), 2.33–2.42 (4H, m, C_3 and C_5 protons of piperazine), 3.90 and 4.24 (4H, two brs, C_2 and C_6 protons of piperazine), 4.67 (2H, s, $COCH_2$), 7.61 and 7.65 (4H, two d (J = 8.5 and 8 Hz), 1,4-disubstituted phenyl protons).

For $C_{14}H_{17}F_3N_2S_2$, calcd.: C, 50.28; H, 5.12; N, 8.38; Found: C, 50.30; H, 5.11; N, 8.36.

MS (ES): $[M+1]^+$: 335.

*4-(Trifluoromethyl)benzyl 4-ethylpiperazine-1-carbodithioate (**2g**)*

^{1}H NMR (δ ppm) (DMSO-d_6): 1.00 (3H, t, CH_3), 2.34 (2H, q, N–CH_2), 2.42 (4H, brs, C_3 and C_5 protons of piperazine), 3.89 and 4.24 (4H, two brs, C_2 and C_6 protons of piperazine), 4.67 (2H, s, $COCH_2$), 7.61 and 7.65 (4H, two d (J = 8 and 8.5 Hz), 1,4-disubstituted phenyl protons).

For $C_{15}H_{19}F_3N_2S_2$, calcd.: C, 51.70; H, 5.50; N, 8.04; Found: C, 51.72; H, 5.49; N, 8.03.

MS (ES): $[M+1]^+$: 349.

*4-(Trifluoromethyl)benzyl 4-phenylpiperazine-1-carbodithioate (**2h**)*

^{1}H NMR (δ ppm) (DMSO-d_6): 3.26–3.32 (4H, m, C_3 and C_5 protons of piperazine), 4.07 and 4.39 (4H, two brs, C_2 and C_6 protons of piperazine), 4.71 (2H, s, $COCH_2$), 6.81 (1H, t, (J = 7 and 7.5 Hz), C_4 proton of *N*-phenyl), 6.94 (2H, d (J = 8 Hz), C_3 and C_5 protons of *N*-phenyl), 7.24 (2H, t (J = 8 and 8 Hz), C_2 and C_6 protons of *N*-phenyl), 7.62 and 7.68 (4H, two d (J = 8 and 8 Hz), 1,4-disubstituted phenyl protons).

For $C_{19}H_{19}F_3N_2S_2$, calcd.: C, 57.56; H, 4.83; N, 7.07; Found: C, 57.55; H, 4.85; N, 7.07.

MS (ES): $[M+1]^+$: 397.

*4-(Trifluoromethyl)benzyl 4-(4-methoxyphenyl)piperazine-1-carbodithioate (**2i**)*

^{1}H NMR (δ ppm) (DMSO-d_6): 3.13 (4H, brs, C_3 and C_5 protons of piperazine), 3.69 (3H, s, OCH_3), 4.06 and 4.39 (4H, two brs, C_2 and C_6 protons of piperazine), 4.70 (2H, s, $COCH_2$), 6.84 and 6.92 (4H, two d (J = 9 and 9 Hz), 1,4-disubstituted *N*-phenyl protons), 7.62 and 7.68 (4H, two d (J = 8 and 8.5 Hz), 1,4-disubstituted phenyl protons).

For $C_{20}H_{21}F_3N_2OS_2$, calcd.: C, 56.32; H, 4.96; N, 6.57; Found: C, 56.30; H, 4.97; N, 6.55.

MS (ES): $[M+1]^+$: 427.

*4-(Trifluoromethyl)benzyl 4-(2-pyrimidinyl)piperazine-1-carbodithioate (**2j**)*

^{1}H NMR (δ ppm) (DMSO-d_6): 3.85–3.91 (4H, m, C_3 and C_5 protons of piperazine), 4.04 and 4.35 (4H, two brs, C_2 and C_6 protons of piperazine), 4.70 (2H, s, $COCH_2$), 6. 69 (1H, t (J = 5 and 4.5 Hz) C_4 proton of pyrimidine), 7.64 and 7.68 (4H, two d (J = 8.5 and 8.5 Hz), 1,4-disubstituted phenyl protons), 8.40 (2H, d (J = 5 Hz), C_1 and C_3 protons of pyrimidine).

For $C_{17}H_{17}F_3N_4S_2$, calcd.: C, 51.24; H, 4.30; N, 14.06; Found: C, 51.25; H, 4.29; N, 14.07.

MS (ES): $[M+1]^+$: 399.

AChE/BuChE inhibition

All compounds were subjected to a slightly modified method of Ellman's test [23] in order to evaluate their potency to inhibit AChE and BuChE. The spectrophotometric method is based on the reaction of released thiocholine to give a colored product with a chromogenic reagent 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB). AChE, (E.C.3.1.1.7 from electric eel, 500 units), BuChE (E.C. 3.1.1.8, from horse serum, 1000 units), and donepezil hydrochloride were purchased from Sigma–Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate, DTNB, potassium hydroxide, sodium hydrogen carbonate, gelatine, acetylthiocholine iodide (ATC), and butyrylthiocholine iodide (BTC) were obtained from Fluka (Buchs, Switzerland). Spectrophotometric measurements were performed on a 1700 Shimadzu UV-1700 UV–Vis spectrophotometer.

The anticholinesterase activity of the compounds **2a–j** was measured in 100 mM phosphate buffer (pH 8.0) at 25°C, using ATC and BTC (75 mM) as substrates. In both cases, DTNB (10 mM) was used in order to observe absorbance changes at 412 nm. Donepezil hydrochloride was used as a positive control [24].

Enzymatic assay

Enzyme solutions were prepared in gelatine solution (1%), at a concentration of 2.5 units/mL. AChE or BuChE solution (50 μL) and compound solution (50 μL), which is prepared in 2% DMSO at a concentration range of 10^{-1}–10^{-6} mM, were added to 3.0 mL phosphate buffer (pH 8 ± 0.1) and incubated at 25°C for 5 min. The reaction was started by adding DTNB (50 μL) and ATC (10 μL) to the enzyme-inhibitor mixture. The production of the yellow anion was recorded for 10 min at 412 nm. As a control, an identical solution of the enzyme without the inhibitor was processed following the same protocol. The blank reading contained 3.0 mL buffer, 50 μL 2% DMSO, 50 μL DTNB, and

10 μL substrate. All processes were assayed in triplicate. The inhibition rate (%) was calculated by the following equation:

$$\text{Inhibition } \% = \frac{(A_C - A_I)}{A_C} \times 100$$

where A_I is the absorbance in the presence of the inhibitor, A_C is the absorbance of the control, and A_B is the absorbance of blank reading. Both of the values were corrected with blank-reading value. SPSS for Windows 15.0 was used for statistical analysis. Data were expressed as mean ± SD.

Kinetic characterization of AChE inhibition

Enzyme kinetic characterization study for compound **2g** was performed under same incubation conditions as described above using acetylthiocholine as substrate at various concentrations (0.5, 0.25, 0.125, 0.0625, and 0.03125 μM ATCI) and DTNB was used as chromophoric reagent [25]. A parallel control with no inhibitor in the mixture was used for comparison. Compound **2g** (IC_{50} = 0.53 μM) was analyzed in triplicate; and Lineweaver–Burk (1/*V* vs. 1/[S]) plot was constructed.

Molecular docking study

Docking studies were carried out by Autodock_vina v.1.1 [26]. The X-ray structure of *Tc*AChE in complex with *N*-saccharinohexyl-galanthamine, a derivative showing more than 40 times AChE inhibitory activity higher than that of a known AChE inhibitor, galanthamine [27], was obtained from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do), PDB ID: 3I6Z. All non-protein molecules including water, ions, and the ligand *N*-saccharinohexyl-galanthamine were removed from the pdb structure before docking. MGL Tools v.1.5.4. [28] was used to prepare the ligand and the receptor, which were saved in pdbqt format. Autodock_vina v.1.1 [26] was used to dock the ligand into the binding site of *Tc*AChE and the docking parameters were set as follows: center_x = 1.292, center_y = 65.557, center_z = 65.639, size_x = 40, size_y = 40, size_z = 40, exhaustiveness = 8. Docking of the compound **2g** was performed in a confined grid box defined by MGL Tools v.1.5.4 [28]. Docked ligand were analyzed with MGL Tools v.1.5.4 [28] and the UCSF Chimera-1.7 package [29].

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