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Novel Cholinesterase Inhibitors: Synthesis, *in silico* and *in vitro* Studies

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The synthesis of new functionalized linear diaza and triaza phenothiazine and phenoxazines and their *in silico* and *in vitro* anti-Alzheimer activity is reported. Fifteen new amide derivatives (**8-11 & 13-24**) were synthesized by the reactions of phenothiazines/phenoxazine (**6** or **12**) and various aliphatic and aromatic primary amides (**7**) in the presence of nickel catalyst and anhydrous potassium carbonate under nitrogen atmosphere. The FTIR, ^{1}H NMR, ^{13}C NMR and HR-MS spectra of the synthesized compounds were in agreement with the assigned structures. All the 15 new derivatives were screened for their *in silico* and *in vitro* anti-Alzheimer's activity using the inhibition of acetylcholinesterase and butyrylcholinesterase. The results of the *in silico* experiment showed that most of the synthesized derivatives had good binding energies, binding interaction and bond distances. The most active derivatives in the *in silico* studies was compounds **18** (-12.5 and -11.5 kcal/mol) against acetylcholinesterase and butyrylcholinesterase, respectively. In addition, compound **18** had the best *in vitro* inhibitory activity against acetylcholinesterase and butyrylcholinesterase (99.37% and 82.35%). The results of *in silico* experiment were greatly in agreement with the results of *in vitro* studies. The structure-activity relationship studies revealed that the phenothiazine derivatives had better *in silico* and *in vitro* activities. Furthermore, 2-substitutted phenothiazines had better activity than the unsubstituted phenothiazines. The synthesized compounds showed promising *in silico* and *in vitro* activities against acetylcholinesterase and butyrylcholinesterase and as such could be further developed for the treatment of Alzheimer's disease.

Keywords: Acetylcholinesterase, Alzheimer's disease, Butyrylcholinesterase, Phenoxazines, Phenothiazines.

INTRODUCTION

In spite of the untold hardship brought by Alzheimer's disease, there hasn't been new drug approved for the treatment for the past two decades. Strong evidences have continued to emerge on the role of acetylcholinesterase and butyrylcholinesterase inhibition as a potential target for the treatment of Alzheimer's disease. Acetylcholinesterase (AChE) is a cholinergic enzyme that is located largely at postsynaptic neuromuscular junctions, particularly in muscles and nerves. In normal human epithelial cells and malignancies, the neurotransmitter acetylcholine acts as an autocrine and paracrine growth factor [1,2]. The hydrolysis of acetylcholine to choline, which results in the termination of cholinergic signalling, is the principal function of acetylcholinesterase [3,4]. Acetylcholine is involved in a variety of physiological and pathological cellular processes, including cell proliferation, differentiation, organ development, tissue remodelling, muscle contraction, mucus production and synaptogenesis, all of which occur in the nervous system [4-6]. Inhibition of acetylcholinesterase leads to increased level of acetylcholine and has been linked to the treatment of myasthenia gravis, Alzheimer and Parkinson's disease [7].

Butyrylcholinesterase is the second cholinesterase expressed in mammalian cells and responsible for the hydrolysis of butyryl- and succinyl-choline and aliphatic esters [3,8,9]. This enzyme also acts as an aryl acylamidase and boosts the activity of prote-

ases like trypsin and so inhibiting will also affect protease activity indirectly. It binds to many anti-acetylcholinesterase toxins thereby protecting acetylcholinesterase [10]. Inhibition of butyrylcholinesterase is vital in the therapy of neurodegenerative diseases such as myasthenia gravis, Alzheimer and Parkinson [11].

Phenothiazines are one of the most widely encountered bioactive heterocycles in molecules with pharmacological potential [12,13] and its derivatives have been found to possess fascinating biological applications as antiparkinsonian, anticonvulsant, antidepressant, anti-inflammatory, antimalarial agents amongst others [14-20]. Several authors [8,21-25] have reported varying derivatives of phenothiazine as inhibitors of acetylcholinesterase and butyrylcholinesterase at micro- and nano-molar concentration.

In view of the urgent need for the discovery and development of new drug candidates for the treatment of Alzheimer's disease and the reported potentials of inhibitors of acetylcholinesterase and butyrylcholinesterase in this regard, we report new derivatives of phenothiazines and phenoxazines as good inhibitors of cholinesterases. We chose to exploit aryl *N*-substitution as against the phenothiazine *N*-amides derivatives widely reported in literature. This is to further explore new functionalized derivatives with potential inhibitory activity against acetylcholinesterase and butyrylcholinesterase, the key enzyme target for Alzheimer's disease.

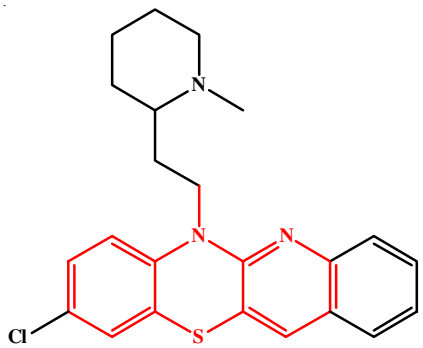
EXPERIMENTAL

All the reactions were carried out under nitrogen atmosphere. Drying of solvents were achieved using molecular sieve 3 Å for 48 h. All reagents were purchased from commercial suppliers like Aldrich, Merck and Fluka, U.S.A. Proton and carbon-13 NMR spectroscopy were run in DMSO-d_6, Brucker 400 MHz at Rhodes University, South Africa. The mass spectral was recorded on Bruker mass spectrometer at University of Copenhagen, Denmark. The melting points were determined using Fisher John's melting point apparatus and are uncorrected. Drying of products were done using hot air oven.

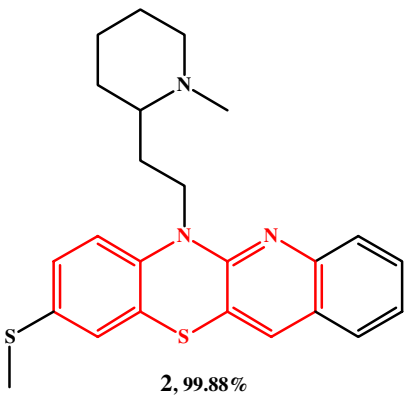
Synthesis of *bis*(triphenylphosphine)nickel(II) chloride: Nickel(II) chloride hexahydrate (2.38 g) in water (2 mL) and diluted with glacial acetic acid (50 mL) and mixed with triphenylphosphine (5.25 g) dissolved in glacial acetic acid (25 mL) and the mixture was kept for 24 h and filtered. The crude product were recrystallized from glacial acetic acid and dried.

Buchwald-Hartwig amidation: Triphenylphosphine (0.013 g, 0.05 mmol) and *bis*(triphenylphosphine)dichloronickel(II) (0.025 g, 0.05 mmol) were placed in a 100 mL three necked flask. Nitrogen gas was introduced for 30 s, 1 mL of water was added and the solution was heated for 2 min at 80 ºC. The preactivation was monitored visually by colour change to black and the resulting catalyst solution was observed. Thereafter, phenoxazine (0.135 g, 0.5 mmol), anhydrous potassium carbonate (0.097 g, 0.7 mmol) and amide (0.7 mmol) in 2 mL of *t*-butanol was added. After 20 min, 2 mL of *t*-butanol was added while the passage of nitrogen gas continued for another 30 s. The entire mixture was refluxed with stirring for 3 h at the temperature of 110 ºC. The resulting product was air dried and crystallized from aqueous ethylacetate (**Scheme-I**).

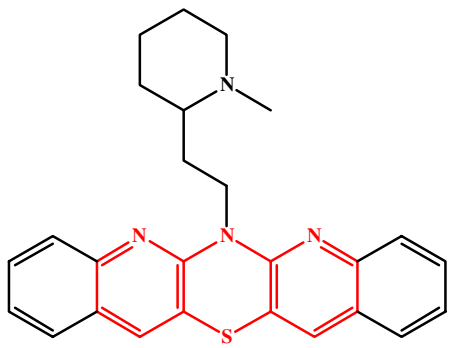
***N*-(12*H*-Benzo[5,6][1,4]oxazino[2,3-*b*]quinoxalin-9-yl)-3-nitrobenzamide (8):** Yield: 83.2%, m.p.: 100-104 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3524, 3463 (NH), 3020 (C=CH), 1680 (C=O), 1625, 1618 (C=N), 1604, 1516, 1460 (C=C), 1538, 1354 (NO_2).



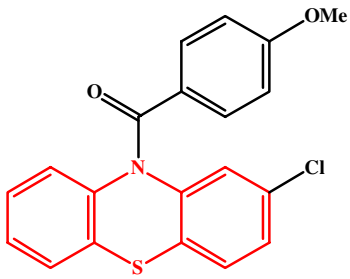
1, 99.88%



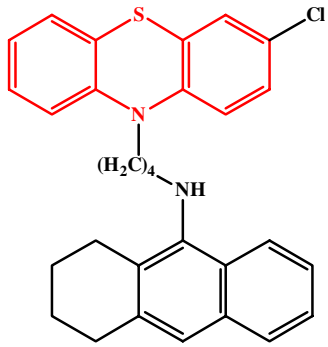
2, 99.88%



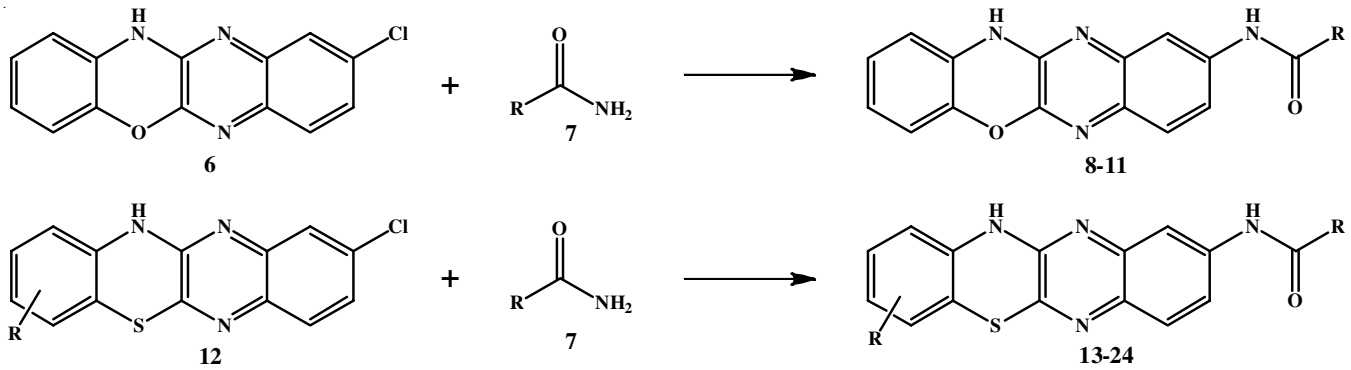
3, 99.76%



4, IC_{50} 5.6 microM



5, IC_{50} 8nM



Scheme-I

^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.32 (s, NH), 8.98 (s, NH), 8.53 (d, 2H, ArH), 8.32 (s, 1H, ArH), 7.93 (d, 1H, ArH), 7.85 (d, 1H, ArH), 7.77 (d, 1H, ArH), 7.01 (d, 1H, ArH), 6.88 (d, 1H, ArH), 6.85 (d, 1H, ArH), 6.77 (d, 1H, ArH), 6.52 (s, 1H, ArH). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 167.14 (C=O), 150.41 (C-NO_2), 150.08, 138.20, 137.08, 136.96, 134.26, 124.41, 123.94, 123.61, 120.40, 119.04, 113.08, 108.66 (13 aromatic carbons). MS (*m/z*): calculated: 399.0968, experimental 400.11.

***N*-(12*H*-Benzo[5,6][1,4]oxazino[2,3-*b*]quinoxalin-9-yl)-benzamide (9):** Yield: 84%, m.p.: 199-200 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3601, 3432 (NH), 3012 (C=CH), 1663 (C=O), 1622 (C=N), 1604, 1485 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.72 (s, 1H, NH of amide), 8.66 (s, 1H, ArH), 8.34 (m, 2H, ArH), 7.43 (d, *J* = 7.89 Hz, 2H, ArH), 7.23 (m, 5H, ArH), 6.83 (m, 2H, ArH), 3.5 (s, 1H, NH of morpholine); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 168.60 (C=O), 158.22, 156.42, 146.11, 140.09, 138.84, 136.91, 136.08, 128.21, 127.17, 124.32, 124.09, 123.89, 123.26, 122.18, 122.01, 120.74, 119.17, 119.04, 112.81. MS (*m/z*): calculated: 354.11.

***N*-(12*H*-Benzo[5,6][1,4]oxazino[2,3-*b*]quinoxalin-9-yl)-acetamide (10):** Yield: 85%, m.p.: 202-203 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3534, 3417 (NH), 3112 (C=CH), 1687 (C=O), 1611 (C=N), 1601, 1485 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.73 (s, 1H, NH), 7.41 (s, 1H, ArH), 7.32 (m, 2H, ArH), 7.21 (m, 2H, ArH), 6.98 (m, 2H, ArH), 3.56 (s, 1H, NH of morpholine), 2.45 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 167.92, 158.46, 151.94, 139.69, 138.24, 137.28, 135.56, 130.02, 129.40, 125.00, 121.65, 120.76, 116.14, 109.52, 24.62. MS (*m/s*): 292.10.

***N*-(12*H*-Benzo[5,6][1,4]oxazino[2,3-*b*]quinoxalin-9-yl)-formamide (11):** Yield: 93%; m.p.: FTIR (KBr, ν_{max}, cm^{-1}): 3668, 3561 (NH), 3116 (C=CH), 2875 (C-H of aldehyde), 1701 (C=O), 1642 (C=N), 1623, 1475 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.68 (s, 1H, CH of aldehyde), 8.05 (s, 1H, ArH), 7.58 (m, 2H, ArH), 7.35 (d, *J* = 8.80 Hz, 1H, ArH), 7.27 (d, *J* = 8.40 Hz, 1H, ArH), 6.85 (m, 2H, ArH), 4.04 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 160.04, 156.48, 151.32, 137.69, 137.18, 131.92, 128.07, 125.57, 125.28, 123.71, 116.19, 115.96. MS (*m/z*): 278.08.

***N*-(12*H*-Benzo[5,6][1,4]oxazino[2,3-*b*]quinoxalin-9-yl)-3-nitrobenzamide (13):** Yield: 76.2%, m.p.: 180-181 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3432, 3385 (NH), 3080 (C=CH), 1687 (C=O), 1650, 1628 (C=N), 1610, 1445 (C=C), 1552, 1261 (NO_2). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.91 (s, 1H, NH of amide), 8.85 (s, 1H, ArH), 8.42 (m, 2H, ArH), 7.73 (d, *J* = 8.16 Hz, 1H, ArH), 7.59 (d, *J* = 8.04 Hz, 2H, ArH), 7.48 (d, *J* = 8.0 Hz, 2H, ArH), 7.33 (d, *J* = 8.17 Hz, 1H, ArH), 7.11 (m, 1H, ArH), 3.79 (s, 1H, NH of thiomorpholine); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 166.27 (C=O of amide), 148.26, 141.20, 140.86, 139.05, 136.21, 135.36, 134.28, 132.50, 131.90, 130.59, 129.31, 129.19, 127.20, 126.45, 126.36, 126.11, 125.76, 123.27, 122.70. MS (*m/z*): 416.07.

***N,N′*-(5*H*-Pyrido[3′,2′:5,6][1,4]thiazinino[2,3-*b*]-quinoxaline-2,8-diyl)*bis*(3-nitro benzamide) (14):** Yield: 86%; m.p.: 360-361 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3553, 3445 (NH), 3120 (C=CH), 1696, 1678 (C=O), 1620 (C=N), 1609, 1481 (C=C), 1502, 1309 (NO_2). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 11.17 (s, 1H, NH), 10.32 (s, 1H, NH), 8.89 (s, 1H, ArH), 8.49 (m, 4H, ArH), 8.12 (s, 1H, ArH), 7.81 (m, 4H, ArH), 7.36 (m, 1H, ArH), 6.88 (m, 1H, ArH), 4.62 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 167.04, 163.74, 148.34, 148.01, 142.02, 138.23, 135.15, 134.43, 133.36, 131.53, 129.72, 127.73, 125.42, 123.27, 123.53, 123.31, 111.13, 108.08. MS (*m/z*): 580.09.

***N*-(2-Methoxy-5*H*-pyrido[3′,2′,:5,6][1,4]thiazino[2,3-*b*]-quinoxalin-8-yl)-3-nitro benzamide) (15):** Yield: 56%, m.p.: 140-141 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3527, 3483 (NH), 3009 (C=CH), 1684 (C=O), 1602, 1454 (C=C), 1522, 1326 (NO_2). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.82 (s, 1H, NH of amide), 8.42 (m, 2H, ArH), 7.86 (d, *J* = 8.02 Hz, 2H, ArH), 7.54 (d, *J* = 8.00 Hz, 2H, ArH), 7.49 (m, 2H, ArH), 6.62 (d, *J* = 8.22 Hz, 1H, ArH), 3.72 (s, 1H, NH of thiomorpholine), 2.63 (s, 3H, CH_3-O); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 166.56 (C=O), 149.19, 134.28, 132.00, 130.54, 129.29, 129.17, 126.35, 122.70, 21.36. MS (*m/z*): 446.08.

***N*-(5*H*-Pyrido[3′,2′:5,6]thiazino[2,3-*b*]quinoxalin-2-yl)-benzamide (16):** Yield: 92%, m.p.: 210-211 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3543, 3476 (NH), 3102 (C=CH), 1656 (C=O), 1612 (C=N), 1604, 1474 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 9.88 (s, 1H, NH of amide), 7.89 (m, 2H, ArH), 7.81 (m, 2H, ArH), 7.58 (d, *J* = 8.22 Hz, 2H, ArH), 7.47 (m, 3H, ArH), 7.16 (m, 2H, ArH), 4.32 (s, 1H of thiomorpholine); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 166.21 (C=O), 161.09 (C=N), 158.12, 151.92, 148.76, 147.88, 142.06, 138.18, 134.65, 134.01, 133.92, 133.41, 132.67, 132.07, 130.11, 129.98, 129.18, 128.29, 124.33, 108.58. MS (*m/z*): 371.08.

***N,N′*-(5*H*-Pyrido[3′,2′:5,6][1,4]thiazino[2,3-*b*]quinoxaline-2,8-diyl)dibenzamide (17):** Yield: 98%, m.p.: 180-182 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3498 (NH), 3040 (C=CH), 1668 (C=O), 1600 (C=N), 1597, 1526, 1484 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.50 (m, 5H, ArH), 7.20 (m, 4H, ArH), 3.5 (s, 1H, NH of thiomorpholine); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 167.88, 159.28, 152.79, 151.79, 132.38, 130.99, 127.59. 126.33, 123.13, 114.19. MS (*m/z*): 490.12.

***N*-(2-Methoxy-5*H*-pyrido[3′,2′:5,6][1,4]thiazino[2,3-*b*]quinoxalin-8-yl)benzamide (18):** Yield: 56%, m.p.: 140-141 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3604, 3484 (NH), 3102 (C=CH), 1685 (C=O), 1631 (C=N), 1616, 1494 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.22 (s, 1H, NH), 7.61 (s, 1H, ArH), 7.58 (m, 5H, ArH), 7.41 (m, 2H, ArH), 7.18 (d, *J* = 8.22 Hz, 2H, ArH), 3.67 (s, 1H, NH of thiomorpholine), 2.91 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 169.22 (C=O), 160.89 (C=N), 158.54 (C=N), 150.62, 144.28, 143.89, 140.09, 138.01, 134.43, 134.02, 129.88, 128.61, 124.43, 124.02, 123.12, 109.54, 22.43. MS (*m/z*): 401.09.

***N*-(5*H*-Pyrido[3′,2′:5,6][1,4]thiazino[2,3-*b*]quinoxalin-2-yl)acetamide (19):** Yield: 48%; m.p.: 220-221 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3585, 3417 (NH), 3001 (C=CH), 1679 (C=O), 1616 (C=N), 1614, 1456 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 10.50 (s, 1H, NH), 7.98 (d, *J* = 8.00 Hz, 1H, ArH), 7.80 (m, 2H, ArH), 7.67 (m, 2H, ArH), 7.04 (d, *J* = 8.00 Hz, 1H, ArH), 3.51 (s, 1H, NH of thiomorpholine), 2.14 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 168.49, 165.26, 150.12, 144.24, 140.11, 139.85, 132.04, 129.76, 126.91, 125.84, 124.69, 123.98, 120.44, 23.86. MS (*m/z*): 309.07.

***N,N′*-(5*H*-Pyrido[3′,2′:5,6][1,4]thiazino[2,3-*b*]quinoxaline-2,8-diyl)diacetamide (20):** Yield: 69%; m.p.: 188-189 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3503, 3427 (NH), 3008 (C=CH), 1680 (C=O), 1601 (C=N), 1598, 1463 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.64 (s, 1H, NH), 7.68 (d, *J* = 8.40 Hz, 2H, ArH), 7.32 (s, 1H, ArH), 7.25 (d, *J* = 8.00 Hz, 1H, ArH), 7.05 (d, *J* = 8.80 Hz, 1H, ArH), 3.78 (s, 1H, NH of thiomorpholine), 2.38 (s, 6H, $2CH_3$); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 164.89, 162.56, 153.07, 151.88, 148.39, 142.06, 132.46, 129.29, 127.34, 126.29, 122.93, 114.45, 111.60, 24.04. MS (*m/z*): 366.09.

***N*-(2-Methoxy-5*H*-pyrido[3′,2′:5,6][1,4]thiazino[2,3-*b*]quinoxalin-8-yl)acetamide (21):** Yield: 80%, m.p.: 212-213 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3612, 3514 (NH), 3102, 3005 (C=CH), 1682 (C=O), 1624 (C=N), 1618, 1464 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 9.59 (s, 1H, NH), 8.04 (s, 1H, ArH), 7.78 (m, 2H, ArH), 6.56 (m, 2H, ArH), 3.64 (s, 3H, CH_3-O), 2.12 (s, 3H, CH_3-C=O); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 169.21, 164.57, 153.26, 149.83, 144.20, 137.82, 132.15, 130.44, 126.54, 124.35, 123.28, 120.10, 114.13, 112.93, 56.41, 23.40. MS (*m/z*) 339.08.

***N,N′*-(5*H*-Pyrido[3′,2′:5,6][1,4]thiazino[2,3-*b*]quinoxaline-2,8-diyl)diformamide (23):** Yield: 98%; m.p.: 178-179 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3618, 3556, 3323 (NH), 3092 (C=CH), 2860 (C-H of aldehyde), 1718, 1712 (C=O), 1624 (C=N), 1599, 1517 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.66 (s, 2H, CHO), 7.65 (m, 2H, ArH), 7.53 (d, *J* = 8.80 Hz, 1H, ArH), 7.35 (m, 1H, ArH), 7.15 (d, *J* = 8.00 Hz, 1H), 3.74 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 161.76, 160.28, 149.95, 146.34, 142.08, 132.38, 127.62, 126.35, 123.22, 114.22, 112.73. MS (*m/z*): 338.06.

***N*-(2-Methoxy-5*H*-pyrido[3′,2′:5,6][1,4]thiazino[2,3-*b*]-quinoxalin-8-yl)formamide (24):** Yield: 84%; m.p.: 261-262 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3622, 3534 (NH), 3048 (C=CH), 2867 (C-H of aldehyde), 1679 (C=O), 1620 (C=N), 1606, 1498 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 8.63 (s, 1H, HC=O), 7.5 (s, 1H, ArH), 7.35 (m, 2H, ArH), 7.12 (d, *J* = 8.40 Hz, 1H, ArH), 6.63 (d, *J* = 8.00 Hz, 1H, ArH), 3.74 (s, 1H of thiomorpholine), 2.62 (s, 3H, CH_3-O); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 158.95, 137.60, 128.59, 127.82, 127.80, 125.66, 124.38, 109.54, 54.06. MS (*m/z*): 325.06.

Protein and ligand preparation: The crystal structure of the target proteins: acetylcholinestrase (PDB: 1ZGC), butyrylcholinestrase (PDB: 4B0O), were retrieved from the protein data bank and prepared using BIOVIA Discovery Studio 2017 R2 version 17.2.0.16349. The protein preparations included deleting of multiple chains, water of crystallization, hetero atoms and addition of polar hydrogens. The Hahn dreiding-like forcefield was applied for the energy minimization and geometry optimization of the proteins. The active binding sites of the proteins were determined by checking the binding site attributes in terms of the X, Y, Z coordinates. The synthesized compounds were drawn using ChemDraw Ultra 12.0 and were converted to their 3D form using Discovery Studio.

Validation of software and docking protocol: Before docking, the BIOVIA Discovery Studio 2017 and the Autodock Vina software used for the docking and the docking protocol were validated by re-docking to determine their suitability and credibility. First, the co-crystallized ligand was removed from the binding cavity of the protein followed by its docking back into the defined active binding site. The best docked pose of the co-crystallized ligand obtained from the docking simulation was compared with the pose before docking in terms of bonding interactions. The amino acid residue interactions were similar, which validates the software as well as the adopted docking protocol.

Molecular docking study: Auto Dock permits the understanding of the molecular interactions between a ligand and a protein in terms of free binding energy and bonding interactions. Molecular docking was carried out to determine the binding energy and bonding interactions of the synthesized compounds with 1ZGC, 4B0O. The synthesized compounds were docked into the active site of the target proteins using Autodock vina. AutodockVina helps in calculating scoring functions used in assigning the binding affinity of a ligand to a protein. During the docking, both the protein and the ligands were regarded as rigid. The docking results were analyzed using BIOVIA Discovery Studio. Interactions such as hydrogen bond, hydrophobic, electrostatic, van der Waal occured between the ligand-protein. Based on the number of hydrogen bond interaction between ligand-protein, a specific ligand pose was selected. A docking score between -7 to -9 is considered good while a docking score < than -10 is considered best. The lower the binding energy, the higher the binding affinity occurs between the ligand-protein [26-31].

In vitro biological activities

***In vitro* acetylcholinesterase inhibitory assay:** Acetylcholinesterase inhibition was assayed using Ellman method [32], using the standard substrate (acetylcholine iodide). Each sample concentration was mixed with 500 µL enzyme solution, incubated at 37 ºC for about 45 min. Absorbance was read at 412 nm after adding 3.5 mL; 0.5 mM acetylcholine, 1 mM DTNB, in 0.05M phosphate buffer (pH 7.20) using JENWAY 6404 spectrophotometer. Assay reactions with synthesized compounds were all performed in duplicate. The percentage enzyme inhibition was calculated as follows:

$$\text{AChe activity } (\%) = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is control absorbance and A_1 is sample absorbance.

***In vitro* butyrylcholinesterase inhibitory assay:** Butyrylcholinesterase inhibition was assayed using Ellman method [32], using the standard substrate (butyrylcholine iodide). Each sample concentration was mixed with 500 µL enzyme solution, incubated at 37 ºC for about 45 min. Absorbance was read at 412 nm after adding 3.5 mL; 0.5 mM butyrylcholine, 1 mM DTNB, in 0.05M phosphate buffer (pH 7.20) using JENWAY 6404 spectrophotometer. Assay reactions with synthesized compounds were all performed in duplicate. The percentage enzyme inhibition was calculated as follows:

$$\text{BChe activity } (\%) = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is control absorbance and A_1 is sample absorbance.

RESULTS AND DISCUSSION

The reactions of appropriate phenothiazines and phenoxazine (**6** or **12**) with aromatic and aliphatic amides (**7**) in the presence of catalytic amount of *bis*-(triphenylphosphine)-dichloronickel(II), potassium carbonate as a base and *t*-butanol as solvent afforded the amide derivatives (**8-11** and **13-24**) in good yields after 4 h refluxing. The compounds were characterized using Fourier transform infrared, proton and carbon-13 nuclear magnetic resonance and mass spectroscopies. The FTIR showed among other bands, the band assignable to carbonyl of amide and N-H stretching vibration. The proton showed the peaks assignable to the N-H of an amide and the R group while the carbon-13 NMR explicitly showed the peaks for the carbons in R and also the carbonyl carbon. The mass spectra showed the peaks for the exact mass of the compounds either in the molecular mass (M^+) or adduct form $(M+X)^+$.

***In silico* bioactivity evaluation:** The *in silico* acetylcholinesterase inhibitory activity (Table-1) revealed that compounds **15**, **17** and **18** as the most active derivative with binding energy of -12.2, 12.0 and 12.5 kcal/mol, respectively. Generally, all the compounds showed the binding affinity ranging from -12.5 to -8.4 kcal/mol while the distances ranged from 1.88 to 3.34 Å. The binding mode and interaction of the two most active derivatives (Figs. 1 and 2) revealed that the amino acid residues interacted with the compound using hydrogen bonding.

The *in silico* butyrylcholinesterase inhibitory activity (Table-1) revealed that compounds **15**, **17** and **9** as the most active derivative with binding energy of -11.1, -10.8 and -10.0 kcal/mol, respectively. Generally, all the compounds showed that binding affinity ranging from -11.1 to -8.4 kcal/mol while the bond distances ranged from 1.92 to 3.08 Å. The binding mode and interaction of the two most active derivatives (Figs. 3 and 4) revealed that the amino acid residues interacted with the compound using hydrogen bonding.

***In vitro* acetylcholinesterase inhibitory activity:** The acetylcholinesterase inhibitory activity result (Table-2) showed that compounds **15**, **17**, **18**, **20** and **21**, as having above 90% inhibition (95.87, 96.50, 99.37, 95.87 and 91.25), respectively. Among the synthesized derivatives inhibited acetylcholinesterase at above 50% except compounds **9** and **13** (40.75 and 13.37%), respectively. All the top five compounds with best *in silico* acetylcholinesterase inhibitory activity also possessed good percentage inhibition in the *in vitro* studies. Interestingly, compound **18** had the best *in vitro* and *in silico* inhibitory properties against acetylcholinesterase. In addition, compounds **15**, **17** and **18** were among the top five best compounds in the *in silico* experiment and correspondingly in the *in vitro* experiment. The best derivative among the phenoxazine derivative was compound **11** with percentage inhibitory activity of 86.87%. Most of the derivatives showed improved activity from the derivatization. In fact, the activity of compound **6** was better than compounds **8** and **9** which were derived from it, suggesting that other substituent on the phenoxazine played a major role in the bioactivity. Compounds **8** and **9** show a considerable

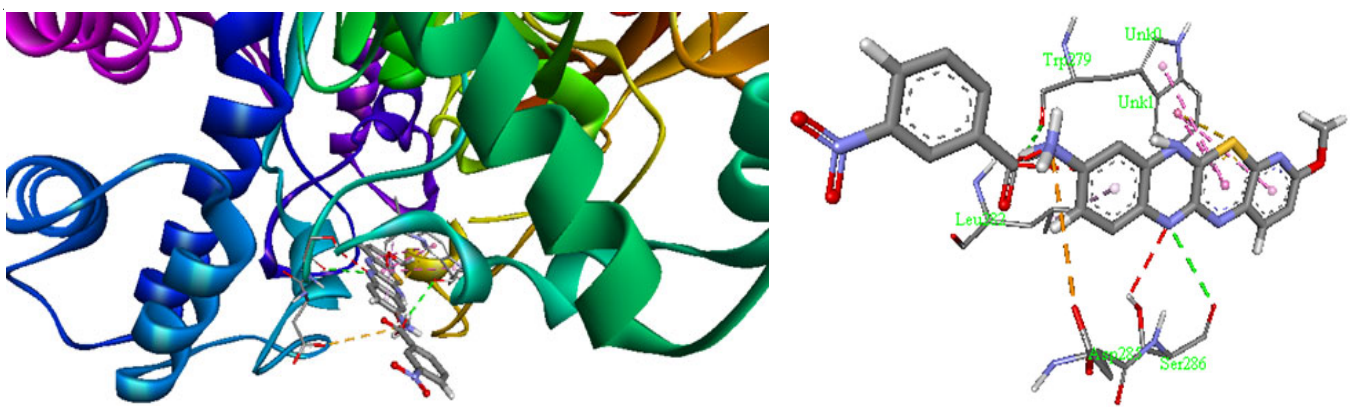


Fig. 1. Binding pose and binding interaction of compound **15** at the active site of acetylcholinesterase 1ZGC

TABLE-1
In silico ACETYLCHOLINESTRASE AND BUTYRYLCHOLINESTERASE ACTIVITY

Compounds	Acetylcholinesterase			Butyrylcholinesterase		
	Binding energy (Kcal/mol)	Amino acid residue	Distance (Å)	Binding energy (Kcal/mol)	Amino acid residue	Distance (Å)
9	-10.6	Ser A: 286	2.45	-10.0	Asp A:375	2.46
		Ser A: 286	2.66		GlnA: 517	2.65
10	-9.4	Ser A: 286	2.93	-8.8	Ala A: 199	2.12
		Asp A: 285	2.05		Gly A: 117	2.37
		Arg A: 289	2.95		Ser A: 287	2.26
11	-8.7	Asn A: 230	3.01	-8.6	Gly A: 117	2.12
		Trp A: 524	2.41		Gly A: 116	2.06
		Asn A: 525	2.45		Ala A: 199	2.36
		His A: 398	3.26		His A: 438	2.66
					Ser A: 287	2.43
15	-12.2	Ser A: 286	3.34	-11.1	Gly A: 116	2.67
		Trp A: 279	2.90		Ala A: 199	2.50
					Ser A: 287	3.01
					Pro A: 285	3.08
16	-10.4	Phe A: 285	2.21	-9.4	Try A:373	2.80
		Ser A: 286	2.24		GlnA: 517	2.43
		Ser A: 286	2.91			
17	-12.0	Asp A: 285	3.02	-10.8	Asp A:375	2.38
		Trp A: 279	2.06		Asp A: 391	2.15
		Asn A: 280	2.62		GluA: 387	2.41
18	-12.5	Ser A: 286	2.39	-11.5	Try A:332	2.90
		Ser A: 286	2.91		AsnA: 289	2.04
		Phe A: 284	2.18		Pro A: 285	2.55
					Pro A: 285	2.61
19	-9.2	Phe A: 288	2.33	-8.8	Gly A: 117	1.94
		Phe A: 284	2.35		Gly A: 116	2.06
		Ser A: 286	2.66		Ala A: 199	2.51
		Ser A: 286	2.91		His A: 438	2.64
20	-9.6	Ser A: 286	1.92	-9.1	Gly A: 117	1.95
		Tyr A: 121	2.80		Gly A: 116	2.17
					Ala A: 199	2.25
					His A: 438	2.78
					Pro A: 285	2.88
					Ser A: 287	2.77
21	-9.2	Ser A:286	2.99	-8.6	Gly A: 117	2.08
		Arg A: 289	2.95		Gly A: 116	2.08
		Asp A: 285	2.12		His A: 438	2.96
					Ser A: 287	2.34
22	-8.5	Glu A:73	2.03	-8.5	Gly A: 117	2.08
		Tyr A: 70	2.86		Gly A: 116	2.14
		Tyr A: 70	2.64		Ala A: 199	2.40
		Tyr A: 334	2.79		His A: 438	2.72
		Tyr A: 334	2.97		Ser A: 287	1.92
					Ser A: 287	2.39
23	-8.4	Ser A: 286	2.55	-8.6	Asn A: 68	2.28
		Ser A: 286	2.79		Gly A: 117	2.32
		Tyr A: 121	2.96		Gly A: 116	2.55
		Asp A: 285	3.02		Ala A: 199	2.19
		Leu A: 282	3.30		His A: 438	2.96
					Ser A: 287	2.12
24	-9.0	Asn A: 230	2.75	-8.4	Pro A: 285	2.91
		Asn A: 525	2.81		Gly A: 117	2.05
		Leu A: 305	1.95		Gly A: 116	2.05
		His A:398	2.14		Ala A: 199	2.26
		Trp A: 524	2.04		His A: 438	2.44
					Ser A: 287	2.72

reduction in activity (55.25 and 40.75%, respectively) when compared to compound **6**. Among the phenothiazine derivatives, only compound **13** (13.37%) had activity lower than the parent phenothiazine (**12**, 67.87%). Structure-activity analysis showed that methoxyphenothiazine derivatives had better activity than the rest of the derivatives. It was observed that having substitution at position-2 enhanced the acetylcholinesterase inhibitory activity.

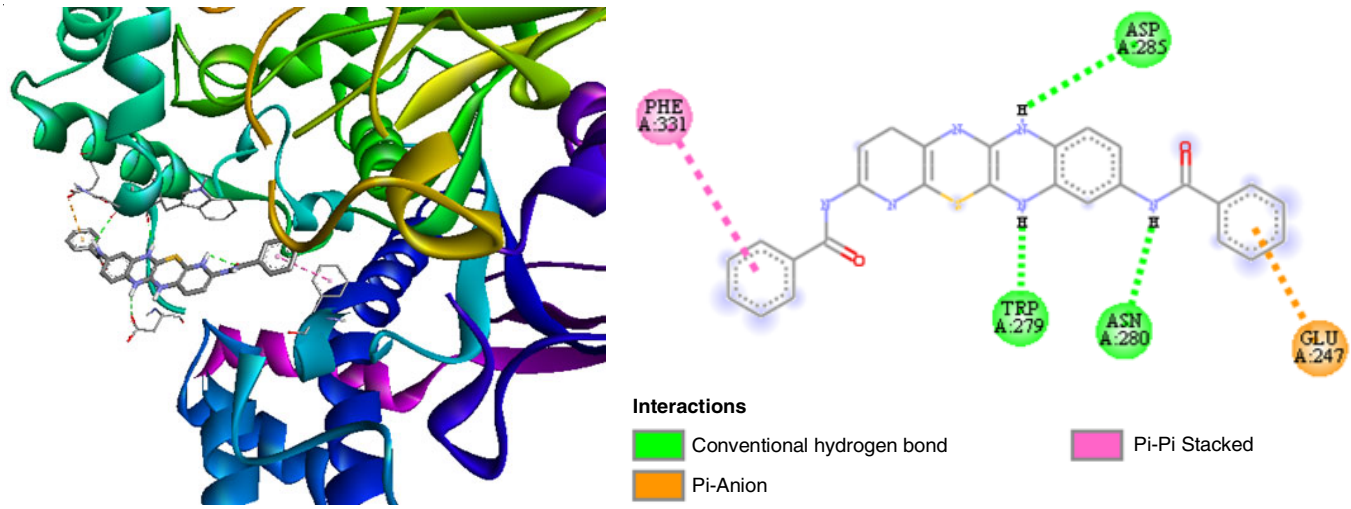


Fig. 2. Binding pose and binding interaction of compound **18** at the active site of acetylcholinesterase 1ZGC

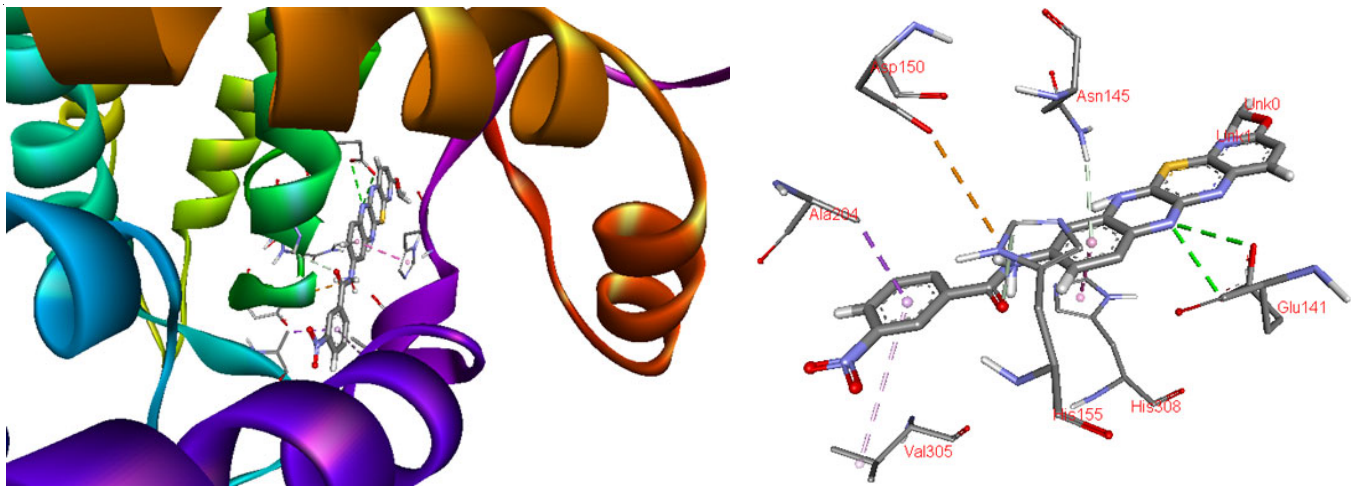


Fig. 3. Binding pose and binding interaction of compound **15** at the active site of butyrylcholinesterase 4B0O

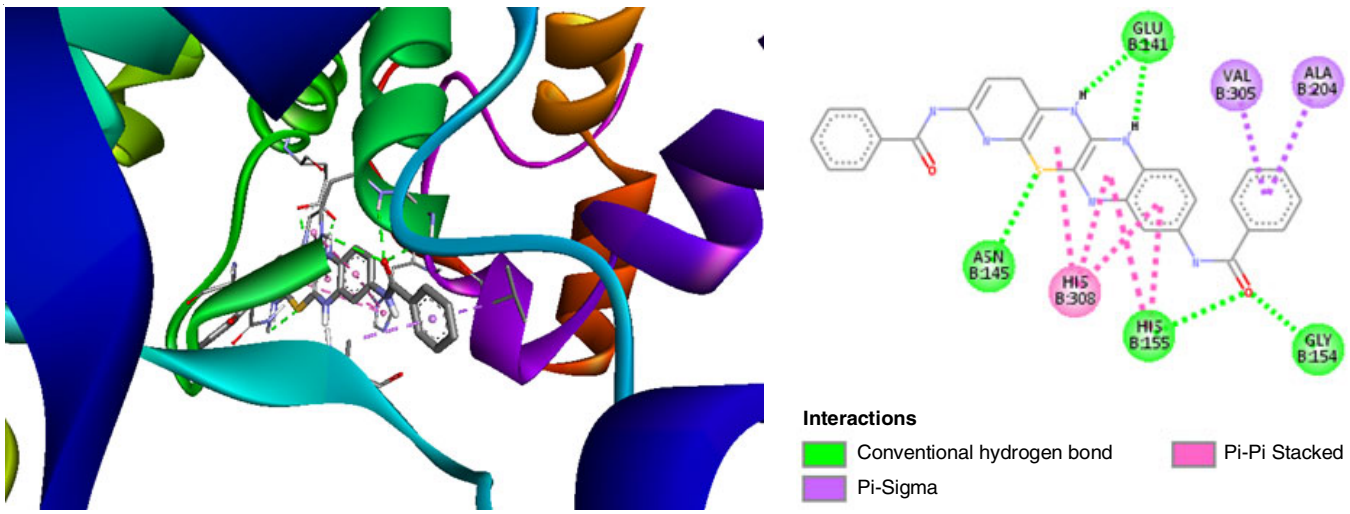


Fig. 4. Binding pose and binding interaction of compound **17** at the active site of butyrylcholinesterase 4B0O

***In vitro* butyrylcholinesterase inhibitory activity:** The results of the inhibitory activity of butyrylcholinesterase (Table-3) indicated that the compounds had fair activity. Only one of the derivatives was able to show > 50% inhibition of butyrylcholinesterase at 5 µM concentration. The *in vitro* results quite agreed with the *in silico* result. Compounds **15**, **17** and **18** showed the lowest binding energies (-11.1, -10.8, and -11.5 kcal/mol) and the highest butyrylcholinesterase inhibitory

TABLE-2
in vitro ACETYLCHOLINESTERASE INHIBITORY ACTIVITY (%)

Samples	ACHE		Samples	ACHE		Samples	ACHE	
	Abs.	Inh. (%)		Abs.	Inh. (%)		Abs.	Inh. (%)
8	0.358	55.25	**15**	0.033	95.87	**21**	0.070	91.25
9	0.474	40.75	**16**	0.175	78.12	**22**	0.085	89.37
10	0.146	81.75	**17**	0.028	96.50	**23**	0.178	77.75
11	0.105	86.87	**18**	0.013	98.37	**24**	0.279	65.12
13	0.693	13.37	**19**	0.159	80.12	**6**	0.174	78.25
14	0.222	72.25	**20**	0.033	95.87	**12**	0.257	67.87
						Blank	0.800	

TABLE-3
in vitro BUTYRYLCHOLINESTERASE INHIBITORY ACTIVITY (%)

Samples	BCHE		Samples	BCHE		Samples	BCHE	
	Abs.	Inh. (%)		Abs.	Inh. (%)		Abs.	Inh. (%)
8	0.686	17.10	**15**	0.543	34.34	**21**	0.629	23.94
9	0.793	4.11	**16**	0.729	11.85	**22**	0.818	1.08
10	0.563	31.92	**17**	0.801	3.14	**23**	0.843	-1.93
11	0.914	-11.00	**18**	0.146	82.35	**24**	0.689	16.68
13	0.698	15.59	**19**	0.668	19.22	**6**	0.465	43.77
14	0.520	37.12	**20**	0.925	-11.85	**12**	0.396	52.11
						Blank	0.827	

activity (82.35%) as shown in Table-1. Among the top five compounds with best *in silico* butyrylcholinesterase inhibitory activity, only compounds **16** and **17** had poor *in vitro* activity of 11.85% and 3.14% as against -9.4 and -10.8 kcal/mol, respectively in the *in silico* experiment. Particularly, compound **18** had the highest molecular docking score and also showed the most activity in an *in vitro* testing. Again, the phenothiazine derivatives showed the better activity than the phenoxazine derivatives. In addition, compound **18** had demonstrated good activity against acetylcholinesterase and butyrylcholinesterase at the *in silico* and *in vitro* studies.

Conclusion

Fifteen new derivatives of phenoxazine and phenothiazine was synthesized and investigated for their *in silico* and *in vitro* acetylcholinesterase and butyrylcholinesterase inhibitory activities. All the reported derivatives had excellent activities against acetylcholinesterase in both the *in silico* and *in vitro* experiment, however, only one of the compounds had good inhibitory activity against butyrylcholinesterase. Compound **18** was found to be the most active compound among the phenothiazines and phenoxazines in the test for the inhibition of acetylcholinesterase and butyrylcholinesterase. Structure-activity relationship studies revealed the phenothiazines as being more active in both the *in silico* and *in vitro* experiment against both acetylcholinesterase and butyrylcholinesterase.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. J.R. Friedman, S.D. Richbart, J.C. Merritt, K.C. Brown, N.A. Nolan, A.T. Akers, J.K. Lau, Z.R. Robateau, S.L. Miles and P. Dasgupta, *Pharmacol. Ther.*, **194**, 222 (2019); https://doi.org/10.1016/j.pharmthera.2018.10.002
2. S.H. Jiang, L.P. Hu, X. Wang, J. Li and Z.G. Zhang, *Oncogene*, **39**, 503 (2020); https://doi.org/10.1038/s41388-019-1006-0
3. N. Halder and G. Lal, *Front. Immunol*., **12**, 660342 (2021) https://doi.org/10.3389/fimmu.2021.660342
4. P.E. Gold, *Neurobiol. Learn. Mem.*, **80**, 194 (2003); https://doi.org/10.1016/j.nlm.2003.07.003
5. I. Esterlis, J.O. Hannestad, F. Bois, R.A. Sewell, R.F. Tyndale, J.P. Seibyl, M.R. Picciotto, M. Laruelle, R.E. Carson and K.P. Cosgrove, *J. Nucl. Med.*, **54**, 78 (2013); https://doi.org/10.2967/jnumed.112.111922
6. M.R. Picciotto, M.J. Higley and Y.S. Mineur, *Neuron*, **76**, 116 (2012); https://doi.org/10.1016/j.neuron.2012.08.036
7. D. Mohammad, P. Chan, J. Bradley, K. Lanctot and N.A. Herrmann, *Expert Opin. Drug Saf.*, **16**, 1009 (2017); https://doi.org/10.1080/14740338.2017.1351540
8. S. Darvesh, R.S. McDonald, A. Penwell, S. Conrad, K.V. Darvesh, D. Mataija, G. Gomez, A. Caines, R. Walsh and E. Martin, *Bioorg. Med. Chem.*, **13**, 211 (2005); https://doi.org/10.1016/j.bmc.2004.09.059
9. O. Lockridge, *Pharmacol. Ther.*, **148**, 34 (2015); https://doi.org/10.1016/j.pharmthera.2014.11.011
10. M. ka, *Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub.*, **155**, 219 (2011); https://doi.org/10.5507/bp.2011.036
11. M. Pohanka, *Int. J. Mol. Sci.*, **15**, 9809 (2014); https://doi.org/10.3390/ijms15069809
12. K. Arentsen, S. Caddick, F.G.N. Cloke, A.P. Herring and P.B. Hitchcock, *Tetrahedron Lett.*, **45**, 3511 (2004); https://doi.org/10.1016/j.tetlet.2004.02.134
13. R. Sharma, P. Samadhiya, S.D. Srivastava and S.K. Srivastava, *Org. Commun.*, **4**, 42 (2011).
14. V. Singh, R. Khanna, V.K. Srivastava, G. Palit and K. Shanker, *Drug Res.*, **42**, 277 (1992).

15. J.N. Domínguez, S. López, J. Charris, L. Iarruso, G. Lobo, A. Semenov, J.E. Olson and P.J. Rosenthal, *J. Med. Chem.*, **40**, 2726 (1997); https://doi.org/10.1021/jm970266p
16. M. Kalkanidis, N. Klonis, L. Tilley and L.W. Deady, *Biochem. Pharmacol.*, **63**, 833 (2002); https://doi.org/10.1016/S0006-2952(01)00840-1
17. A. Rajasekaran and P.P. Thampi, *Acta Pharm. Turcica*, **45**, 227 (2003).
18. P. Archana, K. Rani, V.K. Bajaj, R. Srivastava, A. Chandra and A. Kumar, *Forsch. Drug Res.*, **53**, 301 (2003).
19. Y.S. Sadanandam, M.M. Shetty, A.B. Rao and Y. Rambabu, *Eur. J. Med. Chem.*, **44**, 197 (2009); https://doi.org/10.1016/j.ejmech.2008.02.028
20. N.N. Meghasham, K.G. Mahesh and K.G. Pravin, *World J. Pharm. Res.*, **3**, 1064 (2014).
21. T. Kucukkilinc and I. Ozer, *Arch. Biochem. Biophys.*, **461**, 294 (2007); https://doi.org/10.1016/j.abb.2007.02.029
22. K. Lodarski, J. Joñczyk, N. Guzior, M. Bajda, J. Gladysz, J. Walczyk, M. Jeleñ, B. Morak-Mlodawska, K. Pluta and B. Malawska, *J. Enzyme Inhib. Med. Chem.*, **30**, 98 (2015); https://doi.org/10.3109/14756366.2014.889127
23. G. Tin, T. Mohamed, N. Gondora, M.A. Beazely and P.P.N. Rao, *MedChemComm*, **6**, 1930 (2015); https://doi.org/10.1039/C5MD00274E
24. S. Mpousis, S. Thysiadis, N. Avramidis, S. Katsamakas, S. Efthimiopoulos and V. Sarli, *Eur. J. Med. Chem.*, **108**, 28 (2016); https://doi.org/10.1016/j.ejmech.2015.11.024
25. L. Gorecki, E. Uliassi, M. Bartolini, T. Janockova, M. Hrabinova, B. Monti, J. Pejchal, V. Hepnarova, L. Muckova, J.Z. Karasova, E. Mezeiova, M. Benkova, L. Prchal, T. Kobrlova, O. Soukup, S. Petralla, J. Korabecny and M.L. Bolognesi, *ACS Chem. Neurosci.*, **12**, 1698 (2021); https://doi.org/10.1021/acschemneuro.1c00184
26. D.K. Lahiri, M.R. Farlow, K. Sambamurti, N.H. Greig, E. Giacobini and L.S. Schneider, *Curr. Drug Targets*, **4**, 97 (2003); https://doi.org/10.2174/1389450033346957
27. J. Cheung, M.J. Rudolph, F. Burshteyn, M.S. Cassidy, E.N. Gary, J. Love, M.C. Franklin and J.J. Height, *J. Med. Chem.*, **55**, 10282 (2012); https://doi.org/10.1021/jm300871x
28. S. Auriacombe, J.J. Pere, Y. Loria-Kanza and B. Vellas, *Curr. Med. Res. Opin.*, **18**, 129 (2002); https://doi.org/10.1185/030079902125000471
29. T. Mohamed and P.P.N. Rao, *Curr. Med. Chem.*, **18**, 4299 (2011); https://doi.org/10.2174/092986711797200435
30. B.M. McGleenon, K.B. Dynan and A.P. Passmore, *Br. J. Clin. Pharmacol.*, **48**, 471 (1999); https://doi.org/10.1046/j.1365-2125.1999.00026.x
31. G. Kryger, I. Silman and J.L. Sussman, *Structure*, **7**, 297 (1999); https://doi.org/10.1016/S0969-2126(99)80040-9
32. G.L. Ellman, K.D. Courtney, V. Andres Jr. and R.M. Featherstone, *Biochem. Pharmacol.*, **7**, 88 (1961); https://doi.org/10.1016/0006-2952(61)90145-9