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Evaluation of derivatives of 2,3-dihydroquinazolin-4(1*H*)-one as inhibitors of cholinesterases and their antioxidant activity: *In vitro*, *in silico,* and kinetics studies

OLUWATOYIN BABATUNDE[1], SHEHRYAR HAMEED[1], KINGSLEY ADIBE MBACHU[1,2], FAIZA SALEEM[1], SRIDEVI CHIGURUPATI[3], ABDUL WADOOD[4], ASHFAQ UR REHMAN[4], VIJAYAN VENUGOPAL[5], KHALID MOHAMMED KHAN[1,7]* MUHAMMAD TAHA[7], OLUSEGUN EKUNDAYO[2] and MARIA AQEEL KHAN[6]

[1]H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan; [2]Department of Chemistry, University of Ibadan, Nigeria; [3]Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, Qassim University, Buraydah-52571, Saudi Arabia; [4]Department of Biochemistry, Computational Medicinal Chemistry Laboratory, UCSS, Abdul Wali Khan University, Mardan, Pakistan; [5]Faculty of Pharmacy, AIMST University, Kedah-08100, Malaysia; [6]Third World Center for Science and Technology, H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan and [7]Department of Clinical Pharmacy, Institute for Research and Medical Consultations (IRMC), Imam Abdulrahman Bin Faisal University, P. O. Box 1982, Dammam, 31441, Saudi Arabia

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Abstract: In search of potent inhibitors of cholinesterase enzymes and antioxidant agents, synthetic derivatives dihydroquinazolin-4(1*H*)-one **1-38** were evaluated as potential anti-Alzheimer agents through *in vitro* acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitions and radical (DPPH and ABTS) scavenging activities. The (SAR) was mainly based on the different substituents at the aryl part which showed a significant effect on the inhibitory potential of enzymes and radical scavenging activities. The kinetic studies of most active compounds showed a noncompetitive mode of inhibition for AChE and a competitive mode of inhibition for the BChE enzyme. Additionally, molecular modeling studies were carried out to investigate the possible binding interactions of quinazolinone derivatives with the active site of both enzymes.

Keywords: quinazolinone; dual inhibitors; acetylcholinesterase; butyrylcholinesterase

*Corresponding author E-mail: khalid.khan@iccs.edu, drkhalidhej@gmail.com; Tel.: +922134824910
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INTRODUCTION

In the central nervous system (CNS), one of the preeminent neurotransmitters is acetylcholine (ACh) which is related to memory and cognition. Insufficient ACh levels in the CNS can lead to diseases such as Alzheimer's disease (AD).[1] AD is the most common cause of dementia in elderly people and is characterized by several impaired cortical functions, including judgment, memory loss, comprehension, orientation, language deficit, and learning capacity.[2] The predominant symptoms of all types of dementia are thought to be associated with the gradual decline of broad and compact cholinergic innervation of the human cerebral cortex. This decline contributes to the behavioral and cognitive deficits in AD and is also linked with the reduced levels of neurotransmitters, choline acetyltransferase, acetylcholinesterase (AChE), and ACh.[3] AChE and butyrylcholinesterase (BChE) enzymes are hydrolytic enzymes that act on the neurotransmitter ACh by cleaving it into choline and acetate, thereby stopping their action in the synaptic cleft.[4] Both enzymes are found in amyloid plaques and neurofibrillary tangles in the brain.[5] AChE is the most important enzyme that regulates the level of acetylcholine in a healthy brain, while BChE plays an insignificant role. In AD patients, the AChE activity decreases, BChE activity increases and the ratio between AChE and BChE varies from normal to high levels (0.6-11) in the cortical regions of the brain that affect the disease.[6,7] These observations lead to the concept of dual inhibition, and the most effective treatment approach has been suggested to increase ACh levels and limit cholinergic function by inhibiting AChE and BChE enzymes.

Quinazolinones are extensively explored and are considered important as bioactive synthetic molecules for the development of novel therapeutic agents.[8] Quinazolinone belongs to the *N*-containing fused heterocyclic compounds and is a quinazoline with a carbonyl group in the C_4N_2 ring. There are two isomers possible: 4-quinazolinone and 2-quinazolinone, however, the 4-quinazolinone isomer is more common.[9] These compounds have raised universal concerns due to their broad and pronounced biopharmaceutical activities.[10] Many substituted quinazolinones have a broad range of bioactivities such as antimicrobial, antimalarial, antifungal, antiprotozoal, anticancer, antiviral, anti-inflammatory, anti-tubercular, anticonvulsant, diuretic, acaricidal, muscle relaxant, antidepressant, weedicide, and many other biological activities.[11] Quinazolinone compounds are also used in the syntheses of a variety of functional substances for synthetic chemistry and are also present in various drugs (Figure 1). [12]

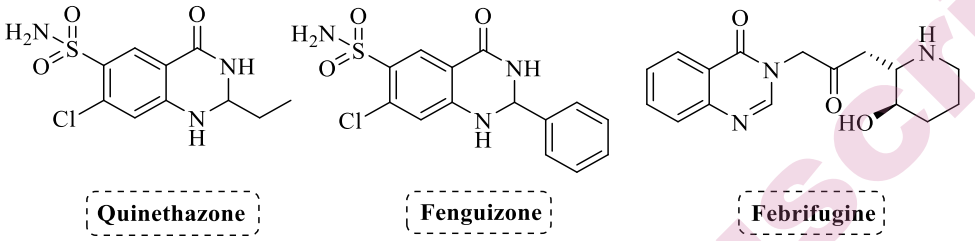


Figure 1. Pharmacological importance of quinazolinone-based drugs

Antioxidant compounds exhibit an important part as a health protection factor.[13] Free radicals are ions, atoms, or molecules possessing an unpaired electron such as hydroxyl, nitric oxide, and superoxide which are called reactive oxygen species (ROS) [14]. ROS are generated in the human body and can damage DNA, proteins, and lipids thus may lead to different complications such as inflammation, toxicity, and carcinogenesis.

Plants-derived antioxidants include carotenes, phytoestrogens, vitamin C, vitamin E, and phytates.[15] Furthermore, chronic diseases which are life-limiting, such as diabetes, cancer, arteriosclerosis, AD, and aging, are developed by radical reactions.[16] Natural or synthetic antioxidant compounds terminate the chain reactions by interacting with free radicals before essential molecules are damaged.[17] Thus, the synthesis of new potent antioxidant compounds is of vital importance for rapidly quantifying the effectiveness of antioxidants in disease prevention.

Our research group is continuously doing efforts in search of lead compounds for two decades to discover new enzyme inhibitors.[18-21] previously, we have explored a large number of potent inhibitors based on quinazoline derivatives, including *α*-amylase, *α*-glucosidase,[22,23] *β*-glucuronidase,[24,] and antileishmanial activities.[25] These heterocycles are reported to possess various significant biological activities. Derivatives of dihydroquinazolin-4(1*H*)-one, in particular, has drawn more and more attention for synthesizing pharmaceuticals and in the field of agrochemicals. Herein we are going to report dihydroquinazolin-4(1*H*)-ones as a new class of inhibitors against acetylcholinesterase, butyrylcholinesterase enzymes, and with its antioxidant potential (Figure 2).

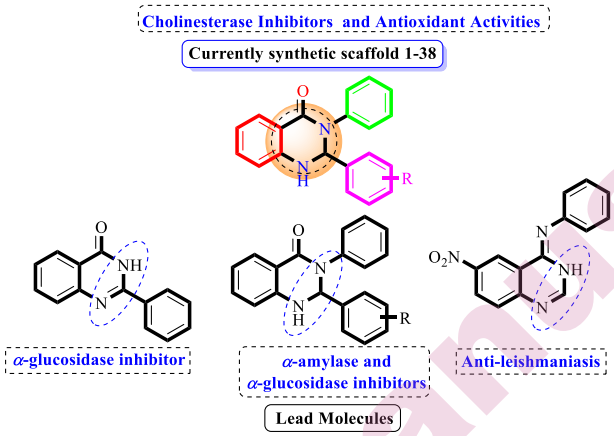


Figure 2. Rationale of the current study

In this study, dihydroquinazolin-4(1*H*)-ones **1-38** (Table I) have been reported as antioxidant agents and potent cholinesterase inhibitors which may improve clinical outcomes for developing anti-AD agents.

Table I. *In vitro* acetylcholinesterase, butyrylcholinesterase activity, and antioxidant activity after using dihydroquinazolin-4(1*H*)-one derivatives **1-38**

Comp. No.	R	AChE activity	BChE activity	DPPH radical activity	ABTS radical activity
		*IC*50 ± SEM[a] / µM)			
1	4-Cl (C_6H_4)	35.04 ± 0.20	37.13 ± 0.18	41.7 ± 0.06	42.97 ± 0.19
2	2-Cl (C_6H_4)	23.08 ± 0.03	26.08 ± 0.43	17.65 ± 0.23	19.47 ± 0.03
3	2,6-Cl (C_6H_3)	24.94 ± 0.12	27.13 ± 0.08	30.7 ± 0.06	32.97 ± 0.19
4	2,4-Cl (C_6H_3)	24.57 ± 0.07	27.57 ± 0.07	16.33 ± 0.02	18.01 ± 0.12
5	2-OH, 3,5-Cl (C_6H_2)	61.89 ± 0.12	67.91 ± 0.18	57.33 ± 0.02	58.01 ± 0.12
6	2-Cl, 6-NO_2 (C_6H_3)	NA[b]	NA[b]	70.7± 0.06	71.97± 0.19
7	5-Cl, 2-OH (C_6H_3)	81.94 ± 0.12	82.13 ± 0.08	83.57 ± 0.17	83.68 ± 0.36
8	3,5-OCH_3 (C_6H_3)	NA[b]	NA[b]	96.65 ± 0.03	94.47 ± 0.13
9	2,5-OCH_3 (C_6H_3)	88.15 ± 0.12	87.15 ± 0.12	84.04 ± 0.02	85.99 ± 0.09
10	2,6-OCH_3 (C_6H_3)	26.94 ± 0.12	27.99 ± 0.09	24.33 ± 0.02	25.01 ± 0.12
11	3,4-OCH_3 (C_6H_3)	87.27 ± 0.18	86.08 ± 0.43	87.57 ± 0.08	89.27 ± 0.18

12	2-Br, 4,5-OCH_3 (C_6H_3)	67.91 ± 0.18	69.02 ± 0.11	51.65 ± 0.03	52.47 ± 0.13
13	2,4-OCH_3 (C_6H_3)	89.7 ± 0.16	85.97 ± 0.19	82.17 ± 0.14	82.01 ± 0.09
14	3,4,5-OCH_3 (C_6H_2)	Na[b]	NA[b]	86.65 ± 0.23	87.47 ± 0.03
15	2,3,4-OCH_3 (C_6H_2)	NA[b]	NA[b]	83.33 ± 0.02	85.01 ± 0.12
16	3-OC_2H_5, 4-OCH_3 (C_6H_3)	27.57 ± 0.07	29.13 ± 0.18	30.04 ± 0.02	31.99 ± 0.09
17	3-OCH_3, 4-OC_2H_5	NA[b]	NA[b]	92.7± 0.06	94.97± 0.19
18	3,5-OCH_3, 4-OH (C_6H_2)	87.27± 0.18	89.7± 0.16	83.46± 0.03	84.61± 0.11
19	4-Br, 3,5-OCH_3 (C_6H_2)	83.08 ± 0.03	84.94 ± 0.12	76.33 ± 0.02	79.01 ± 0.12
20	4-F, 3-OCH_3 (C_6H_3)	51.94 ± 0.12	53.33 ± 0.02	48.65 ± 0.23	49.47 ± 0.03
21	3-Br, 2-OCH_3 (C_6H_3)	89.17 ± 0.16	88.33 ± 0.12	81.7 ± 0.06	85.97 ± 0.19
22	2-F, 4-OCH_3 (C_6H_3)	27.91 ± 0.18	29.02 ± 0.11	31.33 ± 0.12	32.01 ± 0.12
23	2-Cl, 3-OCH_3 (C_6H_3)	88.15 ± 0.12	87.13 ± 0.12	83.04 ± 0.02	84.99 ± 0.09
24	3-OC_2H_5, 2-OH (C_6H_3)	61.01 ± 0.17	64.57 ± 0.07	49.84 ± 0.03	52.71 ± 0.11
25	2-OCH_2(C_6H_5) (C_6H_4)	NA[b]	NA[b]	72.7 ± 0.06	74.97 ± 0.19
26	3-OCH_2(C_6H_5) 4-OCH_3 (C_6H_3)	NA[b]	NA[b]	88.89 ± 0.10	89.09 ± 0.09
27	4-OCH_2(C_6H_5) (C_6H_4)	NA[b]	NA[b]	84.89 ± 0.20	89.09 ± 0.19
28	4-Br (C_6H_4)	25.33 ± 0.02	26.27 ± 0.18	27.33 ± 0.02	28.01 ± 0.12
29	4-CF_3 (C_6H_4)	NA[b]	NA[b]	92.13 ± 0.08	92.79 ± 0.17
30	2-Thiophene	43.08 ± 0.03	46.08 ± 0.43	47.65 ± 0.23	49.47 ± 0.03
31	3-Bromo, 4-OH (C_6H_3)	85.33 ± 0.02	87.47 ± 0.13	83.01 ± 0.07	83.11 ± 0.15
32	4-OCH_3, 3-OH (C_6H_3)	77.27 ± 0.18	75.04 ± 0.52	71.7 ± 0.06	72.97 ± 0.19
33	3-OH (C_6H_4)	47.17 ± 0.15	48.15 ± 0.12	42.33 ± 0.12	43.01 ± 0.12
34	2-OH (C_6H_4)	27.57 ± 0.07	29.02 ± 0.11	28.46 ± 0.03	30.71 ± 0.11
35	4-OH (C_6H_4)	37.7 ± 0.16	38.94 ± 0.12	39.7 ± 0.16	40.97 ± 0.14
36	3,4-OH (C_6H_3)	45.04 ± 0.52	47.7 ± 0.16	48.46 ± 0.03	52.71 ± 0.11
37	2,5-OH (C_6H_3)	77.33 ± 0.02	79.7 ± 0.16	76.65 ± 0.03	77.47 ± 0.13

38	2,3-OH (C_6H_3)	81.94 ± 0.12	83.33 ± 0.02	82.7 ± 0.06	84.97 ± 0.19
Standard= Asc. Acid[c]				15.08 ± 0.03	16.09 ± 0.17
Standard = Donepezil[d]		15.08 ± 0.03	15.08 ± 0.03		

SEM[a] (Standard error of the mean); NA[b] (Not Active); Ascorbic acid[c] (Standard for DPPH and ABTS activities); Donepezil[d] (Standard for AChE and BChE inhibitions).

EXPERIMENTAL

Materials and methods

All enzymes were purchased from Sigma-Aldrich and used without further purification. The acetylcholinesterase enzyme from *Electrophorus electricus* (electric eel) supplied by Sigma-Aldrich (GmbH, USA) whereas butyrylcholinesterase from equine serum procured from Sigma-Aldrich, SRE020, Missouri, USA); 5,5-dithio-bis-nitrobenzoic acid (DTNB), acetylthiocholine iodide 99 % (ATChI), donepezil hydrochloride was obtained from Sigma-Aldrich (United Kingdom). All reagents were purchased from Merck (Germany) and Sigma-Aldrich (USA). Thin-layer chromatography was carried out on precoated silica gel, GF-254 (Merck, Germany). Spots were visualized under ultraviolet light at 254, 366 nm or iodine vapors. EI- and HREI-MS spectra were recorded on MAT 312 and MAT 113D mass spectrometers. The ^{1}H-, ^{13}C-NMR were recorded on Bruker AM spectrometers, operating at 300 and 400 MHz. The chemical shift values are presented in ppm (δ), relative to tetramethylsilane (TMS) as an internal standard, and the coupling constant (J) is in Hz.

Cholinesterase enzyme activity

The *in vitro* AChE and BChE inhibitory activity were measured using the methods described earlier.[26] Briefly, stock solutions (1 mg/mL) of test compounds were prepared using 0.01 % DMSO. Working solutions (0.01 – 100 µg/mL) were prepared by serial dilutions. The various concentrations of test compounds (10 µL) were pre-incubated with sodium phosphate buffer (0.1 M; pH 8.0; 150 µL); AChE solution/ BChE (0.1 U/mL; 20 µL) for 15 min at 25 ˚C and addition of DTNB (10 mM; 10 µL). The reaction was initiated by the addition of ATChI (14 mM; 10 µL). The reaction mixture was mixed using a cyclomixer and incubated for 10 min at room temperature. The absorbance was measured using a microplate reader at 410 nm wavelength against the blank reading containing 10 µL DMSO instead of the test compound. The inhibition was calculated using the formula described in Eq. (1) and the IC_{50} was calculated. Donepezil (0.01–100 µg/mL) was used as the positive control.

$$\text{Inhibition} = ((1\text{-absorbance sample})/\text{absorbance control}) \times 100 \quad (1)$$

Kinetic study assay

In derivatives of 2,3-dihydroquinazolin-4(1*H*)-one, seven compounds **2, 3, 4, 10, 16, 28** and **34** were selected for kinetic studies, based on their lower IC_{50} values (23.08 to 27.57 μM). In kinetic studies, we used acetyl thiocholine iodide (ATCI)/butyrylthiocholine iodide (BTCI) as a substrate at various concentrations (0.175, 0.35, 0.7, and 1.40 mM) and different concentrations of AChE/BChE inhibitors (0, 0.625, 1.25 and 2.5 μM) were used. Enzyme inhibition kinetic mechanisms were determined by using Sigma Plot 14.0 software. The rate of substrate and inhibitor reactions was calculated. Based on the rate of reactions, the software showed the type of enzyme kinetics mechanism. Kinetic studies have shown all the compounds followed as non-competitive type inhibitors (Table I). The types of inhibition of AChE/BChE were determined by Lineweaver Burk plots. The reciprocal of the rate of the reaction was plotted against the reciprocal of substrate concentration to monitor the effect of the inhibitor on both k_m (Michaelis constant the substrate concentration at which the reaction rate is 50% of the Vmax) and V_{max} (In enzyme kinetics, *V*max is the maximum velocity of an enzymatically catalyzed reaction when the enzyme is saturated with its substrate. values.

Radical scavenging assay

DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical activity

Preparation of the DPPH solution was adopted from Molyneux [27] and Blois [28] with minor modifications. All the test compounds were dissolved in 95 % ethanol. Briefly, 0.5 mL of test compounds were added (0 - blank control, 10, 25, 50, 100, 250, 500 and 1000 g/mL) to 0.5 mL of DPPH (2 μM in 95 % ethanol) and the mixture was incubated at room temperature for 30 min. The absorbance was measured at 517 nm,[29] and the percentage inhibition of test compounds was calculated using the following equation using Microsoft Excel software (version 2010). Ascorbic acid was used as the positive control.

$$\text{Scavenging} = ((1\text{-absorbance sample})/\text{absorbance control}) \times 100 \qquad (2)$$

The IC_{50} (half maximal inhibitory concentration) was calculated by constructing a non-linear regression graph between inhibition *vs.* concentration, using Graph Pad Prism software (version 5).[34]

ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) free-radical cation activity

The ABTS free radical cation scavenging ability of the synthesized compounds was determined according to the procedure described earlier.[30] ABTS was dissolved in distilled water (7 mM) and potassium persulphate (2.45 mM) was added. This reaction mixture was left overnight (12 to 16 h) in the dark, at room temperature. Various concentrations of test substances (1000, 500, 250, 100, 50, 25, and 10 μg/mL) were incubated with the $ABTS^+$ solution for 30 min. The absorbance was measured at 734 nm, the inhibition was calculated using the

formula described in Eq. (1) and the IC_{50} was calculated. Ascorbic acid was used as the positive control.

Molecular docking protocol

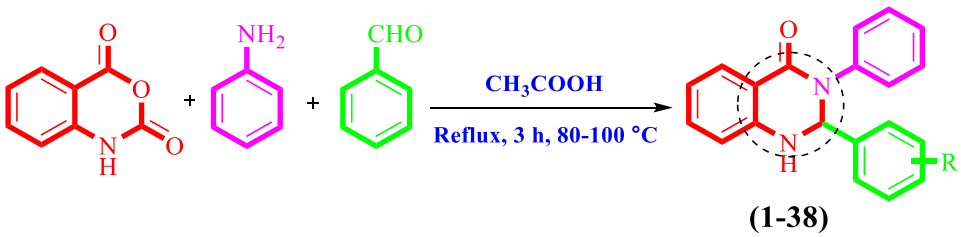
Acetylcholinesterase and butyrylcholinesterase

Molecular docking (MD) was performed using Molecular Operating Environment (MOE)[31] to explore the binding mode of the synthetic compounds against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes. First, the 3D structures for all the compounds were generated using the MOE-builder module. Next, the compounds were protonated, and energy was minimized using the default parameters of the MOE. The structural coordinates for AChE and BChE were retrieved from the protein databank (PDB code; 1acl and 1p0p). All the structure was subjected to MOE for preparation. Further, the protonation was done using the default parameters of the structure preparation module of MOE. Next, the energy was minimized for both coordinates to get minimal energy conformation. Finally, refined structures were used for the docking study using the default parameters of MOE. Before running the docking protocol, we selected a total of often conformations for each compound. The top-ranked conformations based on docking score (S) were selected for protein-ligand interaction (PL) analysis.

RESULTS AND DISCUSSION

Chemistry

Dihydroquinazolin-4(1*H*)-ones **1-38** were synthesized by treating isatoic anhydride, substituted aldehyde, and aniline under reflux for 3 h. The reaction was carried out in acetic acid as a solvent at 80-90 ˚C in Scheme 1. After reaction completion, it was cooled to room temperature. The solution was added to ice water to form a precipitate. The mixture was filtered, and the crude product was washed continuously with an excess of water. The obtained crude product was washed with different solvents to remove impurities, on crystallization from ethanol gave the corresponding pure products having 60-85 % yields.[23] Molecular structures of all compounds **1–38** were identified by EI-MS, HREI-MS, ^{1}H-, and ^{13}C-NMR.



(1-38)

Scheme 1. Synthesis of dihydroquinazolin-4(1*H*)-ones **1-38**

In vitro AChE, BChE inhibitions, and antioxidant activities:

All synthetic dihydroquinazolin-4(1*H*)-ones **1-38** were screened for *in vitro* acetylcholinesterase and butyrylcholinesterase inhibitions, and antioxidant activities. All compounds exhibited good to moderate inhibitory activities in the range of IC_{50} values 23.08-89.7 and 26.01-89.7 µM against AChE, and BChE inhibitions and 16.33-96.65 and18.01-94.97 µM against DPPH and ATBS activities when compared to the donepezil (IC_{50} = 15.08 ± 0.07 µM) and ascorbic acid as the standards (IC_{50} = 15.08 ± 0.07 and 16.09 ± 0.17 µM), respectively (Table I). The structure-activity relationship proposed that all structural features such as benzene ring, carbonyl group, quinazoline moiety, phenyl ring, and aryl ring "R" are taking part in the activity, and due to the presence of different groups "R" at the aryl part significant fluctuation in the activity was observed (Figure S-1 in Supplementary Material).

SAR for AChE and BChE inhibitions and antioxidant activities

Structure-activity relationship (SAR) was discussed for all synthetic compounds which were screened for *in vitro* acetylcholinesterase, butyrylcholinesterase inhibitions, and antioxidant (DPPH and ABTS) activities.

Structure-activity relationship (SAR) for AChE and BChE inhibitory activities

Compounds **1-7**, **28**, and **29** were halogen-substituted including F, Cl, and Br. These compounds displayed inconsistent inhibitory activities against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes. Of these, compound **2** with the *ortho*-chloro substitution was found to be the most potent AChE and BChE inhibitor with IC_{50} values of 23.08 ± 0.03, and 26.08 ± 0.43 µM, respectively. A comparison of the inhibitory activities of compound **2** and compound **1** showed a positional effect on the inhibitory potential. Such in compound **1** the presence of chloro group at *para*-position reduces the inhibitory activity as shown by the IC_{50} values 35.04 ± 0.20 µM for AChE and 37.13 ± 0.18 µM for BChE enzymes. Correspondingly, in compounds **3** (IC_{50} = 24.94 ± 0.12, 27.13±0.08 µM) and **4** (IC_{50} = 24.57 ± 0.07, 27.57 ± 0.07µM), a slight decrease in the inhibitory potential was seen by the addition of chloro groups at the *ortho*-, *para*- and di-*ortho*-positions against AChE and BChE enzymes, respectively. However, the presence of chloro groups in compounds **5-7**, along with other groups such as NO_2 and OH, demonstrated lower potential against AChE and BChE enzymes. *Para*-Bromo substituted compound **28** (IC_{50} = 25.33 ± 0.02, 26.27 ± 0.18 µM), exhibited pronounced activity against both AChE and BChE enzymes, respectively. However, compound **29** with trifluoromethyl substitution was found to be inactive against both enzymes which indicates that the trifluoromethyl group is not actively involved in the binding interaction to the active site of the enzyme (Figure S-2).

In a recently published quinazolinone derivative [33], cholinesterase inhibitors showed superior inhibitory activity compared to the standard drug tacrine. Among them, halogenated compounds showed potential activity against AChE and BChE enzymes. These results showed similarity with our work in which halogenated compounds showed potential activities as compared to the standard donepezil.

It has been found that the incorporation of methoxy substitutions in compounds **8-17** at different positions of aryl moiety (R) has a varying degree of inhibition. Among them, *ortho*-dimethoxy substituted compound **10** was found significantly active with IC_{50} = 26.94 ± 0.12, 27.99 ± 0.09 µM for AChE and BChE enzymes as compared to its *ortho*, *meta*-dimethoxy derivative compound **9**. Surprisingly, it was found that its *meta*-dimethoxy substituted positional isomer **8** was completely inactive. There might be a possibility that compound **8** attained such a conformation that does not fit well into the active site of the enzyme. However, when methoxy groups are present at the adjacent positions in compound **11** (IC_{50}= 87.27 ± 0.02, 86.08 ± 0.43 µM) a noticeable decline in the activity was observed as compared to compound **12** (IC_{50}= 67.91 ± 0.18, 69.02 ± 0.11 μM), where an additional Bromo group is present at *ortho*-position. The positional isomer of **11** *i.e.,* compound **13** demonstrated weak inhibitory potential against both enzymes. In the case of trimethoxy substituted derivatives (compounds **14** and **15**), a complete loss of activity was observed. This might be due to the steric hindrance and bulkiness of the groups. Compounds **16** with *para*-methoxy and *meta*-ethoxy substitutions displayed considerable inhibitory potential with IC_{50} = 27.57 ± 0.07; 29.13 ± 0.18 µM against acetylcholinesterase and butyrylcholinesterase enzymes, respectively. In contrast, compound **17** was found to be inactive against both enzymes (Figure-S-3). Compounds **18-24** and **32** with the combinations of ethoxy/methoxy and other substitutions such as OH, Cl, F, and Br, exhibited moderate inhibition activities against both enzymes. *ortho*-Fluoro and *para*-methoxy substituted compound **22** was found to have relatively good activity in comparison to its other positional analogs. Compound **18** displayed IC_{50} values of 27.91 ± 0.18, and 27.91 ± 0.18 µM against AChE and BChE enzymes, respectively. In contrast, its positional isomer (compound **20**) exhibited low inhibitory potential with IC_{50} = 51.41 ± 0.12, 53.33 ± 0.02 µM against acetylcholinesterase and butyrylcholinesterase enzymes. The activity of the combination of Cl, Br, and OH with methoxy-substituted compounds **18**, **19**, **21**, **23**, **24,** and **32**, displayed moderate to weak inhibitory activities which indicates that these groups are creating steric hindrance and less binding interaction in the enzyme's active site or their positive mesomeric effect is negatively contributing in the activity (Figure S-4).

Surprisingly, *ortho*, *meta,* and *para* benzyloxy substituted derivatives **25-27** were found to be inactive against acetylcholinesterase and butyrylcholinesterase enzymes. It might be due to bulky groups that do not favorably fit in the active site

of the enzyme, which displayed that the presence of hydrophobic groups on the aryl part more specifically the presence of the benzyloxy group, resulted in the loss of activity profile of compounds **25**, **26** and **27**, respectively. Exceptionally, thiophene-substituted analog **30** showed moderate activity against AChE and BChE enzymes with IC_{50} values of 43.08 ± 0.03 and 46.08 ± 0.43 µM, respectively (Figure S-5). Mono-hydroxyl substituted compounds **31-35** showed good to moderate results against acetylcholinesterase and butyrylcholinesterase enzymes. The activity of five hydroxy-substituted derivatives such as **31-35** was different from each other against both enzymes. However, the structure of all five derivatives is very similar to each other but differ only in the position of hydroxyl at aryl part "R". Amongst them, compound **34** (IC_{50} = 27.57 ± 0.07, 29.02 ± 0.11 µM) has *ortho*-hydroxyl group exhibited better activity against AChE and BChE enzymes as compared with compounds **33** and **35**, respectively, which indicate that groups and position displayed significant role in the enzyme inhibition. However, compounds **31** and **32** with the combination of Bromo and methoxy with a hydroxyl group, respectively, exhibited weak inhibitory activities against AChE and BChE enzymes. This activity pattern demonstrated the involvement of di-substituted hydroxy compounds **36-38**, which also displayed moderate to weak inhibitory activities. Compound **36** (IC_{50} = 45.04 ± 0.52, 47.7±0.16 µM) with *meta*, *para* di-hydroxy substitution showed better activity as compared to compounds **37** and **38** against acetylcholinesterase and butyrylcholinesterase enzymes (Figure S-6).

SAR for DPPH and ABTS radical scavenging activities

Based on (SAR), the variations observed in DPPH and ATBS activities of quinazolinones **1-38** were discussed and compared against standard ascorbic acid with IC_{50} = 15.08 ± 0.03 and 16.09 ± 0.17 µM, respectively. Dichloro-substituted compound **4** showed DPPH (IC_{50} = 16.33 ± 0.02 µM) and ABTS (IC_{50} =18.01 ± 0.12 µM) radical scavenging activities, respectively, and was found to be most active in the series. Its positional isomer (compound **3**) displayed a decline in activity against both radicals. However, mono-substituted compound **2** having chloro group at *meta* position (IC_{50} = 17.65 ± 0.23, 19.47 ± 0.03 µM), showed better DPPH and ABTS radical scavenging activities as compared to its positional isomer **1**. Antiradical activity depends on proton and electron transfer between the radical and the scavenging agent. Here 1,4 disubstituted chloro compounds seem to involve electron transfer and free radical scavenging compared to monosubstituted and 1,3 disubstituted chloro compounds. The addition of hydroxyl and nitro substitution at the aryl ring in compounds **5**, **6**, and **7**, respectively, showed moderate to weak potential against DPPH and ABTS radical scavenging activities. The activity of di-methoxy substituted compounds **8**, **9,** and **11-13** showed a further decrease in the activity as compared to *ortho*-dimethoxy substituted compound **10** which showed enhanced DPPH and ABTS radical

scavenging activities. The addition of the methoxy group in compounds **14** and **15** further reduced the activity (Figure S-7). In the case of compound **16** ($IC_{50} = 30.04 \pm 0.02$, $31.99 \pm 0.99\ \mu$M) *para*-methoxy and *meta*-ethoxy groups showed better activities as compared to compound **17** (Figure S-7). Another combination of methoxy with OH, Br, F, and Cl substitutions in compounds **18-23** showed weak potential against DPPH and ATBS activities. Compounds **25, 26,** and **27** bearing benzyloxy substitution displayed decreased radical scavenging activities against DPPH and ATBS. The incorporation of the Bromo group as "R" in compound **28** with IC_{50} values 27.33 ± 0.02, 28.01 ± 0.12 µM, showed better potential than compound **31**. Mono-hydroxy and di-hydroxy substituted compounds **32**, **33**, **35,** and **38** demonstrated good potential against DPPH and ABTS radical scavenging activities as compared to compound **34**. Compounds **23**, **29**, and **30** showed a further decline in the activities as compared to the standard ascorbic acid (Figure S-7).

Kinetic studies on acetylcholinesterase inhibitors

Kinetic studies on the most active AChE enzyme inhibitors (compounds **2-4**, **10**, **16**, **28**, and **34**) were analyzed to interpret the enzyme inhibition mechanisms by using graph fitting analysis in the Sigma-Plot enzyme kinetic software (Figures S-8A-B).

In 2,3-dihydroquinazolin-4(1*H*)-ones all the seven compounds (**2, 3, 4, 10, 16, 28,** and **34)** V_{max} and K_m (Michelis-Menton constant) were in the range of 60.5 to 79.8 (µM/min)/mg and 3.0 to 3.6 mM respectively (Figure S-8A). The K_i (Dissociation constant) values were confirmed from the Dixon plot by plotting the reciprocal of the rate of reaction against different concentrations of compounds, where K_i values of all eight compounds were in the range of 5.0 to 5.9 µM (Figure S-8B). In the uncompetitive type of inhibition, only V_{max} values are affected, and no changes in the K_m value. The low V_{max} and no effect in the K_m value of these compounds indicated an uncompetitive type of inhibition (Table II).

Table II. Kinetic studies of active compounds for acetylcholinesterase inhibition (uncompetitive type of inhibition)

Compound No	V_{max} (µM/min)/mg	K_m / mM	K_i / µM
2	79.8 ± 1.2	3.2 ± 0.01	5.2 ± 0.1
3	70.4 ± 1.0	3.6 ± 0.02	5.4 ± 0.2
4	60.5 ± 2.2	3.0 ± 0.01	5.5 ± 0.5
10	66.8 ± 1.8	3.3 ± 0.02	5.8 ± 0.1
16	71.0 ± 1.2	3.1 ± 0.01	5.0 ± 0.2
28	65.4 ± 1.0	3.2 ± 0.02	5.3 ± 0.1
34	53.2 ± 2.2	3.4 ± 0.01	5.9 ± 0.2
Donepezil	62.0 ± 1.0	3.0 ± 0.01	5.1 ± 0.1

Kinetic studies on butyrylcholinesterase inhibition

Kinetic studies on the most active AChE enzyme inhibitors compounds **2-4**, **10**, **16**, **28**, and **34** were analyzed to interpret their inhibition mechanisms (Figure S-9). In 2,3-dihydroquinazolin-4(*1H*)-ones the V_{max} and K_m of all the seven compounds were in the range of 80.3 to 85.4 (μM/min)/mg and 3.1 to 31.8 mM respectively (Figure S-9A). The K_i values were confirmed from the Dixon plot by plotting the reciprocal of the rate of reaction against different concentrations of compounds, where K_i values of all the five compounds were in the range of 10.3 to 10.9 μM (Figure S-9B). In the competitive type of inhibition, only k_m values are affected and there are no changes in the V_{max} value. The high k_m and no effect in the V_{max} of these compounds indicated a competitive type of inhibition (Table III).

Table III. Kinetic studies of active compounds for butyrylcholinesterase inhibition (competitive type of inhibition)

Compound No	V_{max} (μM/min)/mg	K_m / mM	K_i / μM
2	82.0 ± 2.2	3.1 ± 0.2	10.6 ± 0.5
3	80.3 ± 2.7	9.2 ± 0.1	10.4 ± 0.3
4	82.2 ± 5.3	20.2 ± 0.2	10.7 ± 0.2
10	85.4 ± 1.2	2.1 ± 0.1	10.6 ± 0.4
16	82.0 ± 1.4	3.7 ± 0.2	10.4 ± 0.1
28	84.1 ± 2.4	31.8 ± 0.1	10.3 ± 0.1
34	82.5 ± 2.9	4.7 ± 0.1	10.9 ± 02
Donepezil	80.1 ± 1.6	13.5 ± 0.1	10.2 ± 0.1

Molecular docking studies

AChE and BChE molecular docking study

MD was performed to explore the binding mode of the synthesized compounds against the targeted enzyme (AChE and BChE). MD results are in good agreement with experimental results. We have noticed that compounds bearing the electron-withdrawing groups (EWGs) showed the best inhibitory activity against both targets. Interestingly, as compared with the other activity (*α*-amylase and *α*-glucosidase),[23] we have noted that the compounds bearing 1,3-dichlorobenzene showed high inhibitory potency as compared to 1-chlorobenzene. Similarly, the following compounds showed invert phenomena in the activity against both targets. Those compounds bearing 1-chlorobenzene/1-bromobenzene substitution were found to be active. The PLI profile was enlisted for all docked compounds in Tables S-I and S-II in the Supplementary Material.

Acetylcholinesterase (AChE) molecular docking study

The docking results for most active compound **2** against AChE revealed that the 3-methyl-tetrahydro pyrimidine-4(1*H*)-one moiety of the compound adopted several favorable interactions with catalytic residues (Figure 3A surface representation) including acidic residue Glu72, hydrophobic side chain Tyr334, Trp279, and Phe331, respectively (Figure 3B**)**.

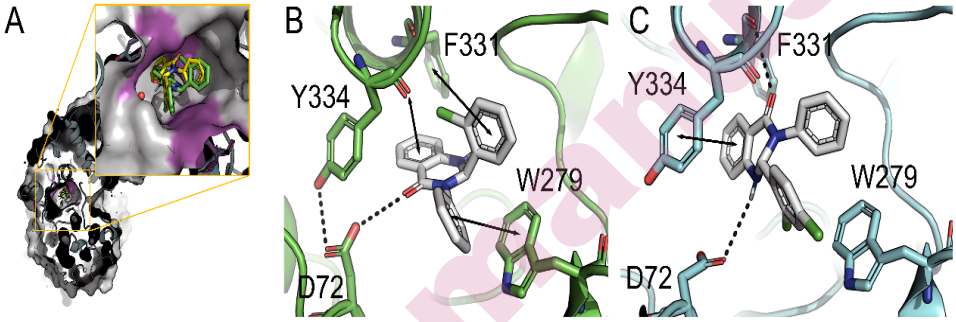


Figure 3A-C. The PLI profile for synthesized compounds against the acetylcholinesterase (AChE) enzymes. **(A)** The surface representation of the enzyme, **(B)** The binding mode of the most potent compound **2** in the series, and **(C)** compound **4**. A double-sided arrow represented the π-stacking

The reason for high potency might include the high number of adopted favorable interactions with catalytic residues. In the case of the 2nd ranked active compound **4**, where the substitution groups are the 2,3-dichloro, a similar interaction was observed. But the only difference so far found is: the active compound adopted π-stacking interaction with the 1-Chloro moiety, whereas it lacks in the 2nd active compound (Figure 3C). This might be one of the reasons for reduced activity in compound **4**. The PLI profile was enlisted for all docked compounds in (Table S-II).

Butyrylcholinesterase (BChE) molecular docking study

In the case of the docking results for most active compounds against BChE (Figure 4A), activity revealed that the compound bearing electron-withdrawing groups (EWG), *i.e.* 1-chlorobenzene (Figure 4B) and 1-bromobenzene (Figure-4C), showed best inhibitory activity against the BChE enzyme. The protein-ligand interaction (PLI) profile for the most active compound **2** and 2nd ranked active compound **28** revealed an interesting observation that both the compound shared similar interaction with the hydrophobic residue Phe329.

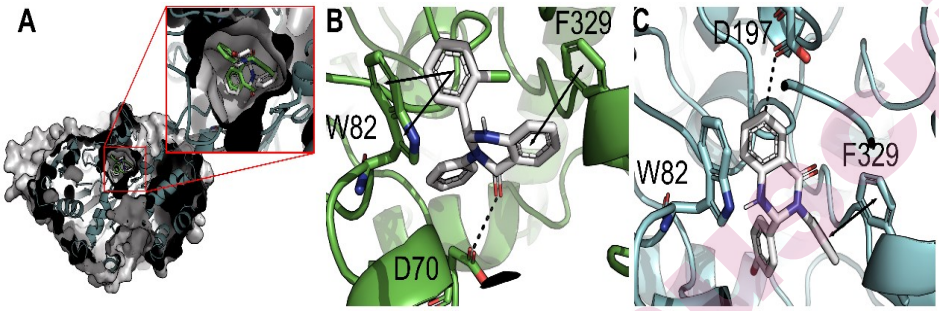


Figure 4A-C. The PLI profile for synthesized compounds against the butyrylcholinesterase (BChE) enzymes. **(A)** The surface representation of the enzyme, **(B)** The binding mode of the most potent compound **2** in the series, and **(C)** Compound **28**. The π-stacking was represented by a double-sided arrow

More interestingly, the most active compound **2** adopted interaction with the acidic residue Glu70 while compound **28** with Glu197, which suggested that might be these two residues play a vital role in enhancing the enzymatic activity. The hydrophobic residue Trp82, which is an active residue in the active site and plays a vital role in the enzymatic activity, adopted two π-stacking interactions with the substituted benzene ring while the compound **28** does not attempt to adapt interaction even though this residue is found in proximity with the 6-ring of the compound.

Overall, these results describe that the compounds bearing the EWG either at *ortho*- or *meta*-position displayed good inhibitory potential against the enzyme while others bearing both *ortho*- and *meta*- or *ortho*- and *para*-positions showed less activity. The PLI profiles were enlisted for all docked compounds in (Table S-II).

CONCLUSION

In the present study, compounds showed moderate to good inhibition against AChE, BChE, and antioxidant activities as compared with the standards donepezil and ascorbic acid, respectively. A structure-activity relationship was also established. *In silico* modeling studies revealed the binding mode of the quinazolinone derivatives. The kinetic studies on the seven most active compounds **2**, **3**, **4**, **10**, **16**, **28**, and **34** were carried out. The compounds **2**, **3**, **4**, **10**, **16**, **28**, and **34** were found to have an uncompetitive mode for acetylcholinesterase enzyme and the compounds **2**, **3**, **4**, **10**, **16, 28**, and **34** were found to be the competitive mode for butyrylcholinesterase enzymes.

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SUPPLEMENTARY MATERIAL

Additional data are available electronically at the pages of the journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/11370, or from the corresponding author on request.

ИЗВОД

ИЗУЧАВАЊЕ ДЕРИВАТА 2,3-ДИХИДРОХИНАЗОЛИН-4(1*H*)-ОН КАО ИНХИБИТОРА ХОЛИНЕСТЕРАЗА И ЊИХОВЕ АНТИОКСИДАТИВНЕ АКТИВНОСТИАКТИВНОСТИ: *In vitro*, *in silico*, И КИНЕТИЧКА ИСПИТИВАЊА

OLUWATOYIN BABATUNDE[1], SHEHRYAR HAMEED[1], KINGSLEY ADIBE MBACHU[1,2], FAIZA SALEEM[1], SRIDEVI CHIGURUPATI[3], ABDUL WADOOD[4], ASHFAQ UR REHMAN[4], VIJAYAN VENUGOPAL[5], KHALID MOHAMMED KHAN[1,7], MUHAMMAD TAHA[7], OLUSEGUN EKUNDAYO[2] и MARIA AQEEL KHAN[6]

[1]H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan; [2]Department of Chemistry, University of Ibadan, Nigeria; [3]Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, Qassim University, Buraydah-52571, Saudi Arabia; [4]Department of Biochemistry, Computational Medicinal Chemistry Laboratory, UCSS, Abdul Wali Khan University, Mardan, Pakistan; [5]Faculty of Pharmacy, AIMST University, Kedah-08100, Malaysia; [6]Third World Center for Science and Technology, H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan u [7]Department of Clinical Pharmacy, Institute for Research and Medical Consultations (IRMC), Imam Abdulrahman Bin Faisal University, P.O. Box 1982, Dammam, 31441, Saudi Arabia

Током истраживања нових активних инхибитора холинестераза и антиоксидативних агенаса, испитивани су синтетички деривати дихидрохиназолин-4(1*H*)-он **1**-**38** као потенцијални агенси за третман Алцхајмерове болести инхибицијом ацетилхолин естеразе (AChE), бутирлихолин естеразе (BChE) и као хватачи слободних радикала (DPPH и ABTS). Доминантан утицај на инхибицију ензима и способност хватања слободних радикала имају супституенти на ароматичном језгру. На основу резултата испитивања кинетике закључено је да једињења делују некопетентивним механизмом инхибиције. Молекулским моделовањем су испитане могуће интеракције током везивања киназолинских деривата у активним местима оба ензима.

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