Acetylcholinesterase inhibition, molecular docking and ADME prediction studies of new dihydrofuran-piperazine hybrid compounds

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Abstract

Novel acrylamide and methacryloyl carrying piperazine-dihydrofuran derivatives (3a-p) were designed and obtained from radical cyclizations of unsaturated piperazine derivatives (1a-f) with 1,3-dicarbonyl compounds (2a-c) mediated by Mn (OAc)3. Obtained compounds were characterized by spectroscopic methods. In vitro AChE inhibitory activities of 3a-p were evaluated against AChE (Acetylcholinesterase) by Ellman method and test results showed that 3a, 3c, 3j, and 3l are the most active AChEIs (AChE inhibitors) of our work with IC50 (half-maximal inhibitory concentration) values of 2.62, 5.29, 1.17, and 3.90 µM, respectively. Furthermore, ligand-protein interactions and inhibitory activity mechanisms of 3a and 3j were investigated by molecular docking. Finally, in silico molecular property and ADME predictions (absorption, distribution, metabolism and excretion) of potential AChEIs were predicted by PreADMET and Molinspiration webservers. It can be concluded that the lead compound 3j show excellent inhibiton and satisfactory druglike characteristics.

Keywords Piperazine · Dihydrofuran · Radical cyclization · Acetylcholinesterase inhibition · Molecular docking · ADME

Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder and is one of the main cause of dementia effecting elderly people [1]. The illness is characterized by executive disorders, memory loss, mood disturbances, depression and progressive loss of cognitive abilities [1, 2]. Based on the report of World Health Organization (WHO) about AD, about 36 million people around globe were suffering from dementia until 2010 and this would be increased to 66 million by 2030 [3]. Many theories were suggested to clarify the exact origin of AD such as cholinergic transmission [4], tau protein hyperphosphorylation [5], and beta-amyloid aggregation [6]. Among them, cholinergic transmission is the most commonly accepted theory and increasing levels of neurotransmitter acetylcholine in the brain is crucial for the treatment of AD [7–9].

Acetylcholinesterase (AChE) is an enzyme in cholinesterase family that catalyzes the rapid hydrolysis of neurotransmitter acetylcholine and terminates impulse transmission at cholinergic synapses [10]. Inhibiting AChE is the most prominent way in the field of AD treatment [11] and there are many commercially available inhibitor drugs such as Donepezil [12], Rivastigmine [13], and Galantamine [14].

Heterocycles bearing nitrogen are important compounds in the field of medicinal chemistry and widely used for their biological properties [15]. Piperazine is considered a privileged structure for its ability of binding to multiple structures with high affinity [16]. Many AChE inhibition studies were performed for piperazine derivatives in literature [17–19].

Dihydrofurans are biologically active heterocycles and useful building blocks for naturally occurring compounds such as Sarcophytoxide [20] and Clerodin [21]. Dihydrofurans can be obtained by C–C bond forming radical cyclization reactions that occur through the addition of α-carbon radicals to unsaturated systems.
studies were conducted to predict druglikeness of obtained compounds, in silico molecular property and ADME (absorption, distribution, metabolism, excretion) prediction mechanisms, the enol form of dimedone (A) reacts with Mn (OAc)₃ and an alpha carbon radical B is formed, while Mn³⁺ reduces to Mn²⁺. Alpha carbon radical can interact with servers.

Chemistry

The reactions of acrylamide substituted piperazines (1a-e) with dimedone (2a) and acetylacetone (2b) were given in Table 1. The reaction of piperazine derivative 1a with dimedone (2a) gave piperazine substituted dihydrofurans 3a (10%) and 3b (45%) from the cyclization of each acyl group and these compounds were differentiated by their ¹H NMR spectra. The ¹H NMR spectrum of 3a shows trans alkene protons at 6.85 and 7.72 ppm as doublet (J = 15.6 Hz) for each proton. Also, geminal protons of dihydrofuran ring can be seen at 2.72 and 3.50 ppm as doublet (J = 15.2 Hz) for each proton. The terminal alkene protons of 3b is resonated at 5.03 and 5.20 ppm as two singlet. Also, vicinal protons of dihydrofuran moeity of 3b can be seen at 4.23 and 6.11 ppm as two doublet (J = 5.6 Hz).

Reactions of piperazine derivatives 1b and 1c with dimedone (2a) gave acrylamide piperazine substituted dihydrofurans 3c (45%) and 3d (60%), respectively. Both radical cyclizations occurred through the methacryloyl group, regioselectively. However, the reaction of methacryloyl and (2E),(4E)-5-phenyl-2,4-pentadienoyl substituted compound 1d with 2a formed compound 3e (20%) through 2,4-pentadienoyl. The exact structure of this compound was clarified with ¹H NMR and HMBC spectra. Also, the reaction of 1e with 2a gave piperazine substituted dihydrofurans 3f (10%) and 3g (40%) from the cyclizations of each acyl group on 1e. In addition, 3h (45%) and 3i (30%) were obtained from the reaction of acetylacetone (2b) with 1a and 1c, respectively.

As can be seen in Table 2, while piperazine-dihydrofurans 3j (13%) and 3k (25%) were obtained from the cyclization (through both acyl groups) of 1a with 2c, 3l (40%) and 3m (50%) were isolated from the cyclization (only through methacryloyl group) of 1b and 1c with 2c. Similarly, while the reaction of 1f with 2c gave piperazine dihydrofurans 3n (20%) and 3o (30%), only 3p (20%) was isolated from the reaction of 1e with 2c.

The proposed mechanism for the formation of dihydrofurans is explained in Scheme 3 [39]. According to this mechanism, the enol form of dimedone (A) reacts with Mn (OAc)₃ and an alpha carbon radical B is formed, while Mn³⁺ reduces to Mn²⁺. Alpha carbon radical can interact with servers.

Results and discussion

Chemistry

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with both unsaturated sides of piperazine compound and both of these pathways (i and ii) are likely to occur at the same time. On pathway i an electron from alkene is added to this \( \alpha \)-carbon radical and produces the radical carbon intermediate C. Intermediate C oxidizes to carbocation D with Mn(OAc)\(_3\) and intramolecular cyclization of D forms the product E. On pathway ii radicalic cyclization reaction follows similar steps on the other unsaturated site and product H is formed.

The main reason behind the regioselectivity of 3b over 3a, 3g over 3f, 3k over 3j and 3o over 3n is due to stability of radical intermediate F over C (Scheme 2). Radical intermediate F is more stable than C, due to aromatic groups adjacent to carbon radical. Because of this reason, 3b, 3g, 3k and 3o which formed through pathway i were obtained regioselectively and in more yields than their counterparts.

**In vitro inhibition results of piperazine-dihydrofuran compounds against AChE**

Over recent years there are some works in literature about AChE inhibition of acrylamide and acrylamide containing piperazine compounds. Pan and coworkers described the synthesis of ferulic acid-memoquin hybrids which contain aromatic acrylamide moieties and evaluated their

| Entry | Piperazines | 1,3-dicarbonyls | Products and yields\(^a\) |
|-------|-------------|-----------------|--------------------------|
| 1     | 1a          | 2a              | 3a, 10%, 3b, 45%         |
| 2     | 1b          | 2a              | 3c, 45%                  |
| 3     | 1c          | 2a              | 3d, 60%                  |
| 4     | 1d          | 2a              | 3e, 20%                  |
| 5     | 1e          | 2a              | 3f, 10%, 3g, 40%         |
| 6     | 1a          | 2b              | 3h, 45%                  |
| 7     | 1c          | 2b              | 3i, 30%                  |

\(^a\) Isolated yields based on 1,3-dicarbonyl compounds.
inhibition capabilities against AChE and reported IC$_{50}$ values between 3.2 and 34.7 µM [40]. Additionally, Shaik and coworkers designed flavone-8-acrylamide compounds and obtained inhibition results between 0.064 and 2.81 µM [41]. Moreover, cinnamic N-benzylpiperidine hybrids were synthesized by Estrada et al. and they obtained good inhibition results (IC$_{50}$ = 0.26–8.73) [42]. Finally, aromatic acrylamide carrying piperazine derivatives were obtained by Singh and coworkers that show AChE inhibition with IC$_{50}$ values between 9.91–29.34 µM [43].

In this work, starting unsaturated piperazine derivatives (1a-f) used in this study were tested against AChE and they show almost no inhibition (IC$_{50}$ > 100 µM). On the other hand, in vitro inhibition capabilities of some of the obtained acrylamide carrying piperezine-dihydrofuran compounds were proved to be significantly high. All results compared to standard drugs Donepezil and presented in Table 3.

IC$_{50}$ values of cinnamoyl acrylamide substituted 3a and methacryloyl substituted 3b were calculated and while 3a has IC$_{50}$ value of 2.62 µM, 3b has almost no inhibition (IC$_{50}$ > 100 µM). Also, IC$_{50}$ values for compounds 3c and

### Table 2 Synthesis of piperazine-dihydrofurans (3j-p)

| Entry | Piperazines | 1,3-dicarbonyls | Products and yields |
|-------|-------------|----------------|--------------------|
| 1     | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| 2     | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| 3     | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
| 4     | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| 5     | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) |

a) Isolated yields based on 1,3-dicarbonyl compounds.

### Scheme 3 Proposed mechanism of Mn(OAc)$_3$ mediated radical cyclization

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IC$_{50}$ values of cinnamoyl acrylamide substituted 3a and methacryloyl substituted 3b were calculated and while 3a has IC$_{50}$ value of 2.62 µM, 3b has almost no inhibition (IC$_{50}$ > 100 µM). Also, IC$_{50}$ values for compounds 3c and
3d are 5.19 and 11.89 µM, respectively. By comparing these results it can be seen that inhibition powers in terms of IC50 align as: 3a > 3c > 3d. This is probably due to increasing steric hindrance. By looking at the unsaturated acrylamide moieties of these structures it can be seen that 3a carries a hydrogen and phenyl group while 3c bears a methyl and phenyl and 3d carries two phenyls. These increasing steric hindrances probably make the inhibitor molecule harder to approach to active site of AChE. In addition, while IC50 value of 3f was calculated as 8.55 µM, it is > 100 µM for methacryloyl containing compound 3g. Similarly, compound 3e shows almost no inhibition (IC50 > 100 µM). It is clear that dihydrofuran-piperazine products that bear aromatic acrylamide moieties have much more inhibition power than the products that carry free methacryloyl group. Also, contrary to products obtained from dimedone, 3h and 3i, which obtained from the reactions of acetylacetone (2b), show no inhibition effects.

Among the piperazine-dihydrofuran compounds obtained from the reactions of ethyl acetooacetate (2c) with acrylamide piperazines (1a-e), compounds 3j (IC50 = 1.17 µM) and 3l (IC50 = 3.90 µM) show the best inhibition effects. Also, IC50 values of 3m, 3n and 3p were calculated as 8.36, 6.11, and 8.42 µM, respectively. By comparing the inhibition powers of 3j, 3l, and 3m it can be seen that inhibition capabilities align as 3j > 3l > 3m. Just like the same reason above increasing steric hindrances decreased the inhibiton powers of these molecules. On the other hand, similar to compounds mentioned above, methacryloyl containing compounds 3k and 3o have almost no inhibition effect (IC50>100 µM). In the light of these informations, it is clear that, aromatic moiety carrying acrylamide substituents on piperazine-dihydrofuran compounds have significantly positive effect on inhibitions. Also, it is concluded that, carboxylate substitution on dihydrofuran group increases inhibition efficiency than other substitutions on dihydrofurans. Based on these results, compound 3j is selected as our lead compound.

Molecular docking results of selected piperazine-dihydrofuran compounds (3a and 3j)

AChE active site is 20 Å deep gorge that is located at the bottom of the enzyme molecule. This active site consists of several subsites. These sites and important residues they contain are; catalytic triad (SER203, HIS447, GLU334), anionic subsite (TRP86, TYR133, GLU202, GLY448, ILE451), oxyanion hole (GLY121, GLY122, ALA204), acyl binding pocket (TRP236, PHE295, PHE297, PHE338) and peripheral anionic subsite (ASP74, TYR124, SER125, TRP286, TYR337, TYR341) [45].

Molecular docking studies were performed on two of our most potent inhibitor compounds (3a and 3j) and Donepezil. Docking procedure was validated by re-docking the native ligand Donepezil to target AChE. Near perfect alignment with a RMSD value of 0.340 was obtained from validation results. Binding score of native ligand Donepezil is −12.2 Kcal/mol. Binding energies for top docking poses of ligands 3a and 3j are −9.6 and −10.4 Kcal/mol, respectively. Superpositioned docking poses of 3a, 3j, and Donepezil in AChE active site cavity can be seen in Fig. 1 and shows good alignment with native ligand Donepezil (Fig. 1).

Ligand-protein interactions of top binding poses of ligands 3a, 3j and Donepezil were given in Fig. 2. By investigating the ligand–protein interactions of Donepezil, it can be seen that N-benzyl moiety of Donepezil made π–π interactions with aromatic groups of HIS447 and TRP86. Also, piperidine ring of Donepezil interacts with

Table 3 IC50 values of piperazine-dihydrofuran compounds (3a-p) and Donepezil towards AChE

| Compound | IC50 ± SD (µM) |
|----------|----------------|
| 3a       | 2.62 ± 0.2     |
| 3b       | >100           |
| 3c       | 5.29 ± 0.5     |
| 3d       | 11.89 ± 1.3    |
| 3e       | >100           |
| 3f       | 8.55 ± 0.6     |
| 3g       | >100           |
| 3h       | >100           |
| 3i       | >100           |
| 3j       | 1.17 ± 0.7     |
| 3k       | >100           |
| 3l       | 3.90 ± 0.5     |
| 3m       | 8.36 ± 0.4     |
| 3n       | 6.11 ± 0.6     |
| 3o       | >100           |
| 3p       | 8.42 ± 0.3     |
| Donepezil | 0.041         |

*aThe values are mean of three independent experiments ± SD.*

Fig. 1 AChE active site cavity with Donepezil (green), 3a (magenta) and 3j (cyan) inside
aromatic moieties of TYR341, TYR337, and PHE338 through π–alkyl and π–σ interactions. In addition, carbonyl oxygen forms a hydrogen bonding with PHE295. Benzene and methoxy groups of Donepezil interact with TRP286 through π–π and π–σ interactions, respectively. Similarly TYR341 residue interacts with benzene and –CH₂ bridge through π–π and π–σ interactions, respectively.

By investigating the docking mode of 3a similar residue interactions with Donepezil can be seen. One of the methyl groups of dimedone ring of 3a forms π–alkyl interactions with aromatic moiety of HIS287. Also, dimedone carbonyl forms a carbon-hydrogen bond with TRP286. Methyl group on dihydrofuran ring interacts with TYR341 through a hydrophobic π–alkyl interaction. Piperazine ring forms hydrophobic π–alkyl interactions with aromatic moieties of TRP286 and carbon-hydrogen bonds with SER293 and TYR341. In addition, acrylamide carbonyl forms hydrogen bondings with PHE295 and ARG296. Finally, aromatic moiety of acrylamide group forms π–π interactions with TYR341 and PHE338.

Also, the lead compound 3j, interacts with similar residues like reference drug Donepezil. It can be seen that ethyl carboxylate moiety forms π–σ interaction with aromatic moiety of TRP86. Also, ester carbonyl forms a Carbon-Hydrogen bond with HIS447. Methyl groups on dihydrofuran ring made π–alkyl interactions with PHE338, TYR124, and PHE297, also one methyl group on dihydrofuran ring interacts with TYR341 through a π–σ interaction. Moreover, carbonyl next to dihydrofuran ring forms a hydrogen bond with TYR124. Additionally, piperazine ring interacts with TYR341 and TRP286 through π–alkyl interactions. Finally, the aromatic ring of cinnamoyl group interacts with LEU289 through a π–alkyl interaction.

By considering the ligand-protein interactions of top binding modes of ligands it can be seen that docking results show similar residue interactions like reference drug Donepezil and support in vitro inhibition results.
In silico molecular property and ADME prediction results

ADME properties are one of the main reasons for a drug candidate to fail in clinical trials. In silico molecular property and ADME predictions of obtained piperazine-dihydrofuran compounds which have inhibition powers against AChE (AChEI’s) (3a, 3c, 3d, 3f, 3j, 3l, 3m, 3n, and 3p) and reference drug Donepezil were carried out using Molinspiration (https://www.molinspiration.com/) and Pre-ADMET (https://preadmet.bmdrc.kr) webservers in order to predict druglikeness of these molecules. According to Lipinski’s rule [46] a drug candidate can possess no more than one violation of the following criteria: (i) Hydrogen bond acceptors must be ≤10. (ii) Hydrogen bond donors must be ≤5 (iii) Molecular weight (MW) must be less than 500 D and (iv) Octanol-water partition coefficient (MiLogP) of the molecule must be ≤5.

As can be seen in Table 4 Piperazine-dihydrofuran compounds show no violation against Lipinski’s rule.

Moreover, in silico ADME prediction results of AChEI’s were given in Table 5 [47].

Human intestinal absorption (HIA) indicates gastrointestinal permeation across membranes for drugs which taken orally. All AChEI compounds show great HIA values over 97%.

In vitro Caco-2 cell permeability is an indication of intestinal absorption of drugs. According to our results all AChEI’s show moderate permeations between 41–53 nm/s.

In vitro MDCK cell permeability test utilizes canine kidney cells to test permeability. All AChEI test compounds show low permeation values.

| Code | MW a | MiLogP b | HBA c | HBD d | Nviole | TPSAf | MVolg |
|------|------|---------|------|-------|---------|-------|-------|
| Rule | <500 | ≤5      | ≤10  | ≤5    | ≤1      | <160 Å | <160 Å |
| 3a   | 422.52 | 3.26   | 6    | 0     | 0       | 66.92 | 398.16 |
| 3c   | 436.55 | 3.14   | 6    | 0     | 0       | 66.92 | 414.72 |
| 3d   | 498.62 | 4.36   | 6    | 0     | 0       | 66.92 | 469.57 |
| 3f   | 442.58 | 3.04   | 6    | 0     | 0       | 66.92 | 405.43 |
| 3j   | 412.49 | 3.15   | 7    | 0     | 0       | 76.16 | 384.68 |
| 3l   | 426.51 | 3.03   | 7    | 0     | 0       | 76.16 | 401.24 |
| 3m   | 488.58 | 4.25   | 7    | 0     | 0       | 76.16 | 456.09 |
| 3n   | 418.51 | 2.87   | 7    | 0     | 0       | 76.16 | 375.39 |
| 3p   | 432.54 | 2.93   | 7    | 0     | 0       | 76.16 | 391.95 |
| Donepezil | 379.50 | 4.10   | 4    | 0     | 0       | 38.78 | 367.89 |

a: Molecular weight (MW); b: logarithm of octanol-water partition coefficient (MiLogP); c: Number of hydrogen bond acceptors (HBA); d: Number of hydrogen bond donors (HBD); e: Lipinski rule violations (nviole); f: Topological polar surface area (TPSA); g: Molecular volume (MVol).

| Code | Human intestinal absorption (%) | In vitro Caco-2 cell permeability (nm/s) | In vitro MDCK cell permeability (nm/s) | In vitro skin permeability (log Kp, cm/h) | In vitro plasma protein binding PPB (%) |
|------|--------------------------------|------------------------------------------|----------------------------------------|------------------------------------------|-----------------------------------------|
| Rule | 0–20 (poor) 20–70 (moderate) 70–100 (well) | <4 (low), 4–70 (moderate), >70 (high) | <25 (low) 25–500 (moderate) >500 (high) | >90 (strongly bound) <90 (weakly bound) |
| 3a   | 98.00 | 49.67 | 0.06 | −3.29 | 82.79 |
| 3c   | 97.86 | 50.32 | 0.06 | −3.25 | 84.87 |
| 3d   | 97.48 | 52.53 | 0.04 | −2.39 | 90.62 |
| 3f   | 99.52 | 53.17 | 0.07 | −3.85 | 84.09 |
| 3j   | 98.95 | 45.52 | 0.11 | −3.17 | 76.39 |
| 3l   | 98.87 | 46.87 | 0.08 | −3.14 | 79.85 |
| 3m   | 97.69 | 50.02 | 0.04 | −2.36 | 89.16 |
| 3n   | 98.65 | 50.25 | 0.17 | −3.75 | 72.94 |
| 3p   | 98.96 | 51.48 | 0.11 | −3.71 | 78.50 |
| Donepezil | 97.95 | 55.52 | 0.14 | −3.04 | 84.61 |
Skin permeability is a factor that indicates delivery of a drug through transdermal administration. All AChEIs’ compounds show negative permeability which shows transdermal administration is not suitable for these molecules.

In vitro plasma protein binding (PPB) indicates percentage of a drug is bound to blood plasma proteins. Our AChE’s show binding values less than 90% except 3d. This means they can efficiently diffuse to cell membranes.

**Conclusion**

In the presented work, new piperazine-dihydrofuran compounds (3a-p) were designed and synthesized from Mn(OAc)₃ mediated radical cyclizations of 1,3-dicarbonyl compounds (2a-c) and acrylamide carrying piperazine derivatives (1a-f) in low and medium yields. AChE inhibition capabilities of starting piperazine derivatives (1a-f) and piperazine-dihydrofuran compounds (3a-p) were tested. Although many of the piperazine-dihydrofuran compounds (3a, 3c, 3d, 3f, 3j, 3l, 3m, and 3p) show inhibition capabilities against AChE, starting acylated piperazine compounds (1a-f) show no inhibition effects. While piperazine-dihydrofuran compounds containing aromatically substituted acrylamide moieties have high inhibition effects against AChE (IC₅₀ values ranging from 1.17 to 11.89 μM), methacryloyl carrying piperazine-dihydrofuran compounds (3b, 3g, 3h, 3i, 3k, and 3o) show almost no inhibitions. Also, carbethoxy substituted piperazine-dihydrofuran compounds show higher inhibition effects than other piperazine-dihydrofurans, especially 3j (IC₅₀ = 1.17 μM) which is our lead compound. In addition, molecular docking studies were performed with the lead compound 3j and the other most potent AChEI 3a to investigate ligand-protein interactions and binding energies. Calculated docking results were compared to standard drug Donepezil. Binding scores of Donepezil is −12.2 Kcal/mol and −9.6, −10.4 Kcal/mol for 3a and 3j respectively. Finally, in silico molecular property analysis and ADME prediction studies show that our lead compound 3j and other AChE’s have satisfactory druglike characteristics. Summarily, the lead piperazine-dihydrofuran compound 3j which carries phenyl substituted acrylamide moiety and carboxylate group have excellent AChE inhibition and satisfactory druglike characteristics. This compound has the potential to be a drug candidate and can be further modified to increase the activity against Acetylcholinesterase.

**Experimental**

All reagents and solvents are commercially available and analytically pure unless otherwise stated. AChE (from electric eel, type V-S), acetylthiocholine iodide (ATCI), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were supplied from Sigma Aldrich. Radical oxidant Mn(OAc)₃ was synthesized by electrochemical method [34].

¹H NMR and ¹³C NMR spectra were recorded on a Varian Mercury-400 High performance Digital FT-NMR and Varian Oxford NMR300 spectrometers. HRMS spectra were obtained on an Agilent 1200/6210 LC/MS spectrophotometer. IR spectra (ATR) were obtained with a Bruker Tensor27 spectrophotometer in the 400–4000 cm⁻¹ range with 2 cm⁻¹ resolutions. UV absorbances were recorded by Rigol Ultra-3000 UV-Vis Spectrophotometer. Melting points were determined on a Gallenkamp capillary melting point apparatus. Thin-layer chromatography (TLC) was performed on Merck aluminum-packed silica gel plates. Purification of products was performed by column chromatography on silica gel (Merck silica gel 60, 40–60 μm) or preparative TLC on silica gel of Merck (PF254-366 nm).

**General synthesis procedure and spectroscopic data of piperazine dihydrofuran compounds (3a-p)**

Starting unsaturated piperazine derivatives (1a-f) were obtained according to our previously reported work [48]. All piperazine-dihydrofuran compounds (3a-p) were synthesized by the general method described below.

[Mn(OAc)₃]₂.H₂O (2 mmol, 0.53 g) in 15 mL glacial acetic was heated to 80 °C until dissolved. After that, the solution temperature was cooled to 65 °C and a solution of 1,3-dicarbonyl compound (2a-c) (1 mmol) and piperazine compound (1a-f) (1.2 mmol) in 3 mL of acetic acid was added. The mixture was stirred and the disappearance of the initial dark brown indicated that the reaction was finished (10-30 min). After that, water was added and the reaction mixture was extracted with CHCl₃ (3 × 20 mL). The combined organic phase was neutralized with saturated NaHCO₃ solution, dried over anhydrous Na₂SO₄, and evaporated. The crude product was purified with column chromatography or preparative TLC (chloroform:acetone (85:15) as eluent).

2-(4-cinnamoylpiperazine-1-carbonyl)-2,6,6-trimethyl-3,5,6,7-tetrahydrobenzofuran-4(2H)-one (3a)

It was obtained as a yellow oil; yield: 10% (42 mg); IR (ATR) νmax 3071, 2961, 2921, 2850, 1725 (C=O), 1652 (C=O), 1630 (C=C), 1226, 1192, 752, 692 (aromatic C-H) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.72 (1H, d, J = 15.6 Hz, H provoke), 7.53 (2H, dd, J = 5.2, 2 Hz, arom. CH), 7.37 (3H, dd, J = 5.2, 2 Hz, arom. CH), 6.85 (1H, d, J = 15.6 Hz, H aryl), 6.8 (1H, d, J = 15.6 Hz, H aryl), 3.87-3.58 (8H, broad), 3.50 (1H, d, J = 15.6 Hz, Ha-3), 2.72 (1H, d, J = 15.2 Hz, Hb-3), 2.30 (2H, d, J = 16.0 Hz), 2.27 (2H, s), 1.63 (3H, s, -CH₃), 1.13 (3H, s, -CH₃), 1.11 (3H, s, -CH₃); ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 194.6
Trans-3-(4-methacryloylpiperazine-1-carbonyl)-6,6-dimethyl-2-phenyl-3,5,6,7-tetrahydrobenzofuran-4(2H)-one (3b)

It was obtained as a yellow oil; yield: 45% (190 mg); IR (ATR) νmax 3054, 2961, 2925, 2868, 1719 (C=O), 1637 (C=O), 1610 (C=C), 1228, 1208, 760, 706 (arom. CH) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.41–7.33 (3H, m, arom. CH), 7.25–7.23 (2H, m, arom. CH), 6.11 (1H, d, J = 5.6 Hz, H-2), 5.20 (1H, s, H_αδελ), 5.03 (1H, s, H_αδελ), 4.23 (1H, d, J = 5.6 Hz, H-3), 4.01–3.30 (8H, broad), 2.47 (2H, d, J = 16.0 Hz), 2.26 (2H, d, J = 16.0 Hz), 1.94 (3H, s, –CH₃), 1.15 (3H, s, –CH₃), 1.04 (3H, s, –CH₃); ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 193.9 (C=O), 178.1 (C=C, C-7a), 171.3 (C=O), 170.3 (C=O), 140.0 (C=C), 139.7, 129.1, 128.9, 125.52, 116.0 (C=C), 112.1 (C=C, C-3a), 90.5, 51.1, 49.9, 47.2, 44.2, 37.9, 34.4, 28.9 (–CH₃), 28.3 (–CH₃), 20.4 (–CH₂); HRMS (ESI) (m/z) Calcd for C₂₅H₂₉N₂O₄ 423.22783 found: 423.22835 (M + H)⁺

Trans-3-(4-methacryloylpiperazine-1-carbonyl)-6,6-dimethyl-2-styril-3,5,6,7-tetrahydrobenzofuran-4(2H)-one (3e)

It was obtained as a yellow oil; yield: 20% (89 mg); IR (ATR) νmax 3067, 2965, 2925, 2854, 1734 (C=O), 1646 (C=O), 1626 (C=C), 1191, 1090, 754, 695 (arom. CH) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.31 (5H, m, arom. CH), 6.66 (1H, d, J = 15.6 Hz, H_αδελ), 6.20 (1H, dd, J = 15.6, 7.6 Hz, H_δελ), 5.74 (1H, t, J = 6.4, H-2), 5.21 (1H, s, H_αδελ), 5.04 (1H, s, H_αδελ), 3.71 (1H, d, J = 6.4 Hz, H-3), 3.98–3.26 (8H, broad), 2.41 (2H, d, J = 17.0 Hz) 2.20 (2H, d, J = 16.4 Hz), 1.96 (3H, s, –CH₃), 1.14 (3H, s, –CH₃) 1.12 (3H, s, –CH₃); ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 193.9 (C=O), 177.7 (C=C, C-7a), 171.3 (C=O), 170.2 (C=O), 140.0 (C=C), 135.3, 134.1, 128.8 (C=C), 128.7, 128.6, 126.8, 125.4 (C=C), 115.9 (C=C), 112.1 (C=C, C-3a), 90.23, 51.06, 47.6, 46.4, 42.3, 37.9, 34.3, 28.7 (–CH₃), 28.4 (–CH₂), 20.4 (–CH₂); HRMS (ESI) (m/z) Calcd for C₂₃H₂₃N₂O₄ 499.25913 found 499.26110 (M + H)⁺

2,6,6-trimethyl-2-(4-(3-phenylbut-2-enoyl)piperazine-1-carbonyl)-3,5,6,7-tetrahydrobenzofuran-4(2H)-one (3c)

It was obtained as a yellow oil; yield: 45% (196 mg); IR (ATR) νmax 3058, 2961, 2921, 2850, 1725 (C=O), 1652 (C=O), 1630 (C=C), 1225, 1192, 752, 692 (arom. CH) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.45 (2H, dd, J = 8.4, 2 Hz, arom. CH), 7.39–7.33 (3H, m, arom. CH), 6.26 (1H, s, H_αδελ), 3.80–3.54 (8H, broad), 3.49 (1H, d, J = 15.2 Hz, H-3a), 2.71 (1H, d, J = 15.2 Hz, H-b3), 2.29 (4H, s), 2.24 (3H, s, –CH₃), 1.62 (3H, s, –CH₃), 1.12 (3H, s, –CH₃), 1.10 (3H, s, –CH₃); ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 194.6 (C=O), 173.0 (C=C, C-7a), 169.75 (C=O), 167.31 (C=C), 141.4 (C=C), 128.6, 128.5, 126.0, 118.8, 116.1 (C=C), 111.4 (C=C, C-3a), 91.9, 50.9, 46.0, 43.7, 37.8, 34.3, 28.7 (–CH₂), 28.6 (–CH₃), 26.3 (–CH₃), 18.06 (–CH₂); HRMS (ESI) (m/z) Calcd for C₂₆H₂₅N₂O₄ 437.24348 found 437.24483 (M + H)⁺

2,6,6-trimethyl-2-(4-(3-thiophen-2-ylbut-2-enoyl)piperazine-1-carbonyl)-3,5,6,7-tetrahydrobenzofuran-4(2H)-one (3f)

It was obtained as a yellow oil; yield: 10% (44 mg); IR (ATR) νmax 3071, 2965, 2921, 2863, 1716 (C=O), 1650 (C=O), 1621 (C=C), 1194, 1017, 759, 692 (arom. CH) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.28 (1H, d, J = 0.8 Hz, arom. CH), 7.21 (1H, dd, J = 4.0, 0.8 Hz, arom. CH), 7.03 (1H, dd, J = 5.2, 4.0 Hz, arom. CH), 6.38 (1H, s, H_αδελ), 3.79–3.55 (8H, broad), 3.50 (1H, d, J = 15.2 Hz, H-3a), 2.72 (1H, d, J = 15.2 Hz, H-b3), 2.34 (3H, s, –CH₃), 2.31 (2H, d, J = 16.0 Hz), 2.25 (2H, s), 1.62 (3H, s, –CH₃), 1.12 (3H, s, –CH₃), 1.11 (3H, s, –CH₃); ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 194.6 (C=O), 172.9 (C=C, C-7a), 171.9 (C=O), 169.7 (C=O), 145.1 (C=C), 127.8, 125.8, 125.6, 116.1 (C=C), 111.5 (C=C, C-3a), 91.9, 50.9, 46.3, 45.8, 37.8, 37.5, 34.2, 29.6 (–CH₂), 28.6 (–CH₃), 26.2 (–CH₃), 17.8 (–CH₃); HRMS (ESI) (m/z) Calcd for C₂₃H₃₀N₂O₄S 443.19990 found 443.20162 (M + H)⁺
3-(4-methacryloypiperazine-1-carbonyl)-2,6,6-
trimethyl-2-(thiophen-2-yl)-3,5,6,7-
tetrahydrobenzofuran-4(2H)-one (3g)

It was obtained as a yellow oil; yield: 40% (177 mg); IR (ATR) v_max 3071, 2956, 2921, 2859, 1736 (C=O), 1643 (C=O), 1610 (C=C), 1194, 1024, 755, 700 (arom CH) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.28 (1H, d, J = 4.4 Hz, arom. CH), 7.00-6.97 (2H, m, arom. CH), 5.22 (1H, s, H₀₁), 5.05 (1H, s, H₀₂), 4.44 (1H, s, H-2), 3.60-3.52 (8H, m), 2.40 (2H, d, J = 16.4 Hz), 2.27 (2H, d, J = 16.4 Hz), 1.95 (3H, s, −CH₃), 1.83 (3H, s, −CH₃), 1.46 (3H, s, −CH₃), 1.21 (3H, s, −CH₃); ¹³C-NMR (100 MHz, CDCl₃, δ (ppm): 194.1 (C=C, C-7a), 171.3 (C=O), 167.94 (C=O), 148.9, 139.9 (C=C), 127.0, 125.4, 123.2, 116.1 (C=C), 112.7 (C=C), 90.8, 53.0, 50.73, 46.28, 42.66, 37.7, 34.6, 28.7 (−CH₃), 28.4 (−CH₃), 23.8 (−CH₃), 20.45 (−CH₃); HRMS (ESI) (m/z) Calcd for C₇₂H₇₀N₂O₄S 443.19990 found 443.20162 (M + H)⁺

1-(4-(4-acetyl-2,5-dimethyl-2,3-dihydrofuran-2-
carbonyl)piperazin-1-yl)-3-phenylprop-2-en-1-one (3h)

It was obtained as a yellow oil; yield: 45% (172 mg); IR (ATR) v_max 3076, 2965, 2912, 2845, 1714 (C=O), 1632 (C=O), 1602 (C=C), 1230, 1022, 756, 708 (arom. CH) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.72 (1H, d, J = 15.2 Hz, H₀₁), 7.54-7.52 (2H, m, arom. CH), 7.41-7.36 (3H, m, arom. CH), 7.86 (1H, d, J = 15.2 Hz, H₀₂), 3.78 (1H, d, J = 14.8 Hz, Ha-3), 3.98–3.56 (8H, broad), 2.79 (1H, d, J = 14.8 Hz, Hb-3), 2.23 (3H, s, −CH₃), 2.20 (3H, s, −CH₃) 1.62 (3H, s, −CH₃); ¹³C-NMR (100 MHz, CDCl₃, δ (ppm): 191.0 (C=O), 177.5 (C=C, C-5), 169.7 (C=O), 165.5 (C=O), 145.7 (C=C), 134.9, 129.9, 128.8, 127.8, 116.3 (C=C), 114.2 (C=C, C-4), 88.6, 46.5, 44.3, 42.0, 29.6 (−CH₃), 26.1 (−CH₃), 24.5 (−CH₃), 14.84 (−CH₃); HRMS (ESI) (m/z) Calcd for C₂₂H₂₀N₂O₄ 383.19653 found 383.19745 (M + H)⁺

Ethyl 5-(4-cinnamoylpiperazine-1-carbonyl)-2,5-
dimethyl-4,5-dihydrofuran-3-carboxylate (3j)

It was obtained as a yellow oil; yield: 13% (51 mg); IR (ATR) v_max 3067, 2969, 2930, 2872, 1736 (C=O), 1643 (C=O), 1608 (C=C), 1194, 1090, 761, 701 (arom. CH) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.71 (1H, d, J = 15.2 Hz, H₀₁), 7.53 (2H, dd, J = 5.2, 2 Hz arom. CH), 7.36 (3H, ddd, J = 5.2, 2 Hz arom. CH), 6.87 (1H, d, J = 15.2 Hz, H₀₂), 4.15 (2H, q, J = 7.2 Hz, −OCH₂CH₃), 3.92-3.55 (8H, broad), 3.61 (1H, d, J = 15.2 Hz, Ha-3) 2.74 (1H, d, J = 15.2 Hz, Hb-3), 2.21 (3H, s, −CH₃), 1.59 (3H, s, −CH₃) 1.27 (3H, t, J = 7.2 Hz, −OCH₂CH₂); ¹³C-NMR (100 MHz, CDCl₃, δ (ppm): 170.5 (C=C, C-2), 165.7 (C=O), 165.5 (C=O), 164.5 (C=C), 143.5 (C=C), 134.9, 129.8, 129.0, 128.8, 127.8, 125.4, 116.4 (C=C), 102.4 (C=C, C-3), 88.3, 59.7, 46.2, 43.5, 42.2, 26.0 (−CH₃), 14.3 (−CH₃), 14.1 (−CH₃); HRMS (ESI) (m/z) Calcd for C₂₁H₁₈N₂O₄S 343.07232 found 343.07230 (M + H)⁺

Trans-Ethyl 4-(4-methacryloypiperazine-1-
carbonyl)-2-methyl-5-phenyl-4,5-dihydrofuran-3-
carboxylate (3k)

It was obtained as a yellow oil; yield: 25% (105 mg); IR (ATR) v_max 3026, 2966, 2930, 2870, 1740 (C=O), 1638 (C=O), 1610 (C=C), 1200, 1025, 750, 700 (arom CH) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.42-7.33 (3H, m, arom. CH), 7.29-7.26 (2H, m, arom. CH), 5.67 (1H, d, J = 7.2 Hz, H-5), 5.21 (1H, s, H₀₁), 5.02 (1H, s, H₀₂), 4.35 (1H, d, J = 7.2 Hz, H-4), 4.15 (2H, q, J = 7.2 Hz, −OCH₂CH₃), 3.80-3.30 (8H, broad), 2.35 (3H, s, −CH₃), 1.94 (3H, s, −CH₃) 1.26 (3H, t, J = 7.2 Hz, −OCH₂CH₃); ¹³C-NMR (100 MHz, CDCl₃, δ (ppm): 171.7 (C=C, C-2), 171.2 (C=O), 169.5 (C=O), 165.0 (C=O), 139.9 (C=C), 139.7, 129.0, 128.8, 127.8, 125.4, 116.0 (C=C), 103.65 (C=C, C-3), 87.3, 59.9, 46.1, 42.0, 20.4 (−CH₃), 14.56 (−CH₃), 14.43 (−CH₃); HRMS (ESI) (m/z) Calcd for C₂₂H₂₀N₂O₄ 413.20710 found 413.20919 (M + H)⁺

Ethyl-2,5-dimethyl-5-(4-(3-phenylbut-2-enoyl)
piperazine-1-carbonyl)-4,5-dihydrofuran-3-
carboxylate (3l)

It was obtained as a yellow oil; yield: 40% (170.8 mg); IR (ATR) v_max 3054, 2961, 2916, 2868, 1732 (C=O), 1696
(C=O), 1617 (C=C), 1228, 1097, 750, 706 (arom. CH) cm⁻¹; 
¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.45 (2H, dd, J = 8.4, 1.6 Hz, arom. CH), 7.36 (3H, m), 6.26 (1H, s, Holey), 4.16 (2H, q, J = 7.2 Hz, -OCH₂CH₃), 3.91-3.50 (8H, broad), 3.61 (1H, d, J = 15.2 Hz, Ha-4), 2.73 (1H, d, J = 15.2 Hz, Hb-4), 2.29 (3H, s, -CH₃), 1.58 (3H, s, -CH₃), 1.27 (3H, t, J = 7.2 Hz, -OCH₂CH₃); ¹³C-NMR (100 MHz, CDCl₃) δ (ppm): 171.5 (C=O), 144.1 (C-1), 128.5, 125.9, 118.8 (C=C), 102.3 (C=C-3), 88.3, 59.7, 46.3, 43.4, 41.2, 26.0 (-CH₃), 26.0 (-CH₃), 17.83 (-CH₂CH₃), 14.0 (-CH₂CH₃); HRMS (ESI) (m/z) Calcd for C₃₂H₃₀N₂O₅S 497.18815 found 497.18714 (M + H)⁺

**Ethyl 5-(4-(3-diphenylacryloyl)piperazine-1-carbonyl)-2,5-dimethyl-4,5-dihydrofuran-3-carboxylate (3 m)**

It was obtained as a yellow oil; yield: 50% (244 mg); IR (ATR) \( \nu_{max} \) 3054, 2961, 2921, 2863, 1730 (C=O), 1650 (C=O), 1620 (C=C), 1228, 1060, 760, 703 (arom. CH) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.37-7.27 (10H, m, arom. CH), 6.30 (1H, s, Holey), 4.15 (2H, q, J = 6.8 Hz, -OCH₂CH₃), 3.52 (1H, d, J = 15.2 Hz, H-4), 3.75-2.76 (8H, broad), 2.66 (1H, d, J = 15.2 Hz, H-4), 2.15 (3H, s, -CH₃), 1.50 (3H, s, -CH₃), 1.25 (3H, t, J = 6.8 Hz, -OCH₂CH₃); ¹³C-NMR (100 MHz, CDCl₃) δ (ppm): 172.8 (C=O, C=C), 170.3 (C=O), 167.7 (C=O), 165.6 (C=O), 140.5 129.5, 128.8, 128.4, 128.1, 120.0, 110.0 (C=C), 102.3 (C=C, C=C), 88.2, 59.7, 46.1, 45.6, 41.1, 25.9 (-CH₂), 14.3 (-CH₃), 14.0 (-CH₃); HRMS (ESI) (m/z) Calcd for C₃₂H₳₂₈N₂O₅S 497.18815 found 497.18714 (M + H)⁺

**Ethyl-2,5-dimethyl-5-(4-(3-thiophen-2-yl)but-2-enoyl)piperazine-1-carbonyl)-4,5-dihydrofuran-3-carboxylate (3p)**

It was obtained as a yellow oil; yield: 20% (86 mg); IR (ATR) \( \nu_{max} \) 3085, 2987, 2930, 2863, 1734 (C=O), 1694 (C=O), 1620 (C=C), 1194, 1062, 761, 706 (arom. CH) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.20 (1H, dd, J = 3.6, 0.8 Hz, arom. CH), 7.03-7.01 (2H, dd, J = 4.8, 3.6 Hz, arom. CH), 6.37 (1H, s, Holey), 4.16 (2H, q, J = 7.2 Hz, -OCH₂CH₃), 3.89-3.50 (8H, broad), 3.59 (1H, d, J = 15.2 Hz, Ha-4), 2.72 (1H, d, J = 15.2 Hz, Hb-4), 2.33 (3H, s, -CH₃), 2.26 (3H, s, -CH₃), 1.58 (3H, s, -CH₃), 1.27 (3H, t, J = 7.2 Hz, -OCH₂CH₃); ¹³C-NMR (100 MHz, CDCl₃) δ (ppm): 170.5 (C=C, C=C), 167.6 (C=O), 165.6 (C=O), 141.5 (C=C), 140.6, 127.8, 126.9, 125.8, 116.25 (C=C), 102.3 (C=C, C=C), 88.3, 59.7, 46.3, 43.5, 41.2, 26.0 (-CH₃), 17.83 (-CH₂CH₃), 14.36 (-CH₂), 14.07 (-CH₂); HRMS (ESI) (m/z) Calcd for C₃₂H₳₂₈N₂O₅S 493.17917 found 493.18051 (M + H)⁺

**Method of in vitro AChE inhibition experiments**

Slightly modified Ellman method was carried out to determine in vitro AChE inhibitory activities of test compounds [49].

The assay solution was prepared by adding 1480 µL of phosphate buffer (pH = 8.0, 0.1 M), 50 µL of DTNB solution (prepared with pH 7 phosphate buffer), 20 µL of test compounds at desired concentration in ethanol-deionized water (1:1), 10 µL of substrate solution (ATCl, in deionized water) and 25 µL of AChE solution (prepared with
deionized water and 1% gelatin). After that assay solution was incubated for 10 min. at 30 °C and absorbance at 412 nm was determined.

A control solution containing all compounds except inhibitor was performed same as above and the absorbance at 412 nm was considered 100% enzyme activity.

The percentage activity of AChE for any tested compound at desired concentration was calculated with the formula:

\[
\text{% enzyme activity} = \left( \frac{A_s}{A_0} \right) \times 100
\]

\(A_s\) : Absorbance of assay solution with inhibitor.

\(A_0\) : Absorbance of control solution.

The concentration of each test compound was tested in triplicate and IC_{50} values were calculated graphically using GraphPad Prism 8.0.3 software. IC_{50} value is defined as the concentration of sample which performs 50% inhibition towards AChE.

Methods of in silico molecular docking experiments

Three dimensional structure of recombinant human AChE complexed with Donepezil was obtained from the Protein Data Bank (4EY7) [50]. B-chain, water molecules, and detergents were removed. Conformational analysis of inhibitor test compounds were performed with Avogadro software and most stable conformations were optimized with semiempirical PM6 method in Gaussian 09 Software. All ligand-protein docking calculations were performed as a semiempirical PM6 method in Gaussian 09 Software. All and most stable conformations were optimized with bitor test compounds were performed with Avogadro software. Conformational analysis of inhibitor was performed same as above and the absorbance at 412 nm was determined.

Methods of in silico molecular docking experiments

Three dimensional structure of recombinant human AChE complexed with Donepezil was obtained from the Protein Data Bank (4EY7) [50]. B-chain, water molecules, and detergents were removed. Conformational analysis of inhibitor test compounds were performed with Avogadro software and most stable conformations were optimized with semiempirical PM6 method in Gaussian 09 Software. All ligand-protein docking calculations were performed as a flexible ligand in rigid protein using AutoDock Vina software [51]. Best docking mod of ligand in terms of binding energy (Kcal/mol) was selected and used.

According to these results, it can be seen that all piperazine-dihydrofuran AChEI compounds present satisfactory druglike properties, including our lead compound 3j.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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