Novel 2-phenylbenzofuran derivatives as selective butyrylcholinesterase inhibitors for Alzheimer’s disease

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Alzheimer’s disease (AD) is a neurodegenerative disorder representing the leading cause of dementia and is affecting nearly 44 million people worldwide. AD is characterized by a progressive decline in acetylcholine levels in the cholinergic systems, which results in severe memory loss and cognitive impairments. Expression levels and activity of butyrylcholinesterase (BChE) enzyme have been noted to increase significantly in the late stages of AD, thus making it a viable drug target. A series of hydroxylated 2-phenylbenzofurans compounds were designed, synthesized and their inhibitory activities toward acetylcholinesterase (AChE) and BChE enzymes were evaluated. Two compounds (15 and 17) displayed higher inhibitory activity towards BChE with IC50 values of 6.23 μM and 3.57 μM, and a good antioxidant activity with EC50 values 14.9 μM and 16.7 μM, respectively. The same compounds further exhibited selective inhibitory activity against BChE over AChE. Computational studies were used to compare protein-binding pockets and evaluate the interaction fingerprints of the compound. Molecular simulations showed a conserved protein residue interaction network between the compounds, resulting in similar interaction energy values. Thus, combination of biochemical and computational approaches could represent rational guidelines for further structural modification of these hydroxy-benzofuran derivatives as future drugs for treatment of AD.

Alzheimer’s disease (AD) is a progressive neurodegenerative brain disorder, named after German psychiatrist Alois Alzheimer. AD is the most common cause of dementia, accounting for up to 80% of all dementia cases, as well as being a major cause of death worldwide1–3. It is common in elderly people over 65 years old and exhibits heterogeneous distribution across the globe, being most prevalent in Western Europe and North America, while less prevalent in Sub-Saharan Africa region4.

Being a multifactorial neurodegenerative brain disorder, the exact pathophysiology of AD is not yet entirely known5. However, several pathogenesis of AD have been suggested: deficits in the cholinergic system6,7, accumulation and deposits of beta-amyloid outside the neurons in the brain8, oxidative stress9 and inflammation10. Early studies performed on patients suffering from AD7 found an altered cholinergic activity, which resulted in cognitive and functional symptoms. In the present study, we focus our attention on the cholinergic system, which is the most cited potential mechanism11,12. The cholinergic system directly contributes to regulation and memory process, thus represents a suitable target for the AD drug design3,13,14. In the cholinergic system disruption in the

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levels of acetylcholine (ACh) is caused by hydrolytic action of cholinesterases (ChEs)15. ACh is a neurotransmitter that plays a role in the modulation of memory function in normal and neurodegenerative conditions16.

Butyrylcholinesterase (BChE) and Acetylcholinesterase (AChE) belong to ChEs family of enzymes and play a role in ACh regulation and in the cholinergic signalling17. The two enzymes are extraordinarily efficient and are able to cleave more than 10000 ACh molecules per second18. AChE is substrate specific in nature and is found in high concentrations in the brain, while BChE is non-specific and is distributed throughout the body19. In particular, it is primarily found in the liver, pancreas and associated with glial and endothelial cells in the brain17,19. In a healthy brain, the AChE enzyme dominantly degrades ACh while BChE plays only a supportive role. The two enzymes display diverse kinetic characteristics depending on ACh concentrations. At low ACh concentrations, AChE’s activity becomes highly dominant, while BChE is more efficient in the hydrolysis at high ACh concentrations20. Initial studies underestimated the importance of BChE in human brain owing to its low expression20. However, other studies have shown the importance of BChE within the nervous system to be pivotal in the late stages of AD21. Indeed, in patients with AD, BChE activity progressively increases, while AChE activity remains unchanged. Moreover, BChE knockouts experiments performed on mouse models demonstrated the role of BChE to maintain the cholinesterase function even in the absence of AChE22.

Despite being encoded by different genes on human chromosomes 7 and 323, at molecular level the two enzymes AChE and BChE share nearly 65% sequence homology. The availability of several X-ray crystallographic structures for the two enzymes24–26 further revealed the similarity of the tertiary structure and particularly the architecture of the active site. The active site consists of a catalytic triad (Ser, His, Glu) and a choline-binding pocket buried nearly 20 Å deep into the surface of the enzymes23. The main difference between the two enzymes is located in the acyl-binding pocket, which accommodates the acyl moiety. In detail, two bulky amino acids (Phe) in AChE are replaced with two smaller amino acids; Val and Leu, thus allowing BChE accommodate large and chemically different molecules.

A well-documented strategy towards an effective management of AD is by developing inhibitors that suppress the ChEs enzymes from breaking down ACh and therefore increasing both the level and duration of the neurotransmitter action13. Current Food and Drug Administration (FDA) approved cholinesterase inhibitors namely: donepezil, rivastigmine and galantamine, help only in controlling the symptoms of AD and do not treat the underlying disease or delay its progression. In this scenario, a continuous research related to development of more potent and highly efficacious cholinesterase inhibitors becomes even more essential.

Heterocyclic ring compounds are known to display broad biological, medicinal and pharmacological characteristics and thus form an important moiety to construct inhibitors against ChEs enzymes. Among them, benzofuran derivatives, since synthesized for the first time by Perkin27 in 1870, has been constantly explored in the synthesis of BChE potent and selective inhibitors. In this context, we recently developed a series of 2-phenylbenzofuran derivatives 34, which exhibited selective inhibitory property for BChE enzyme and with an inhibition IC50 value similar to that of galantamine (~30 μM). It was noted that the contemporary presence of a single hydroxyl group in the para position of the phenyl ring and a halogen substitution at position 7 of the benzofuran scaffold improved the inhibitory activity towards BChE22.

To further elucidate the importance of hydroxyl group substitution in the phenyl-ring we synthesized a series of 2-phenylbenzofuran derivatives alternatively with either two or three hydroxyl substituents in the phenyl-ring with a contemporary presence of either chlorine or bromine at position 7 of the benzofuran scaffold. We performed biological evaluation of the synthesized compounds against Electrophorus electricus AChE (EeAChE) and equine BChE (eqBChE). For the most potent compounds, we also investigated their inhibition activity against human BChE (hBChE). Furthermore, we employed molecular dynamics (MD) simulations to identify key structural and dynamical aspects that influence the inhibitory activity of the potent compounds against hBChE enzyme.

Results

Synthesis of 2-phenylbenzofuran derivatives. All compounds were efficiently synthesized employing Wittig reaction according to the protocol outlined in Fig. 1. The desired Wittig reagents were readily prepared from the conveniently substituted ortho-hydroxybenzyl alcohol a–g28–39 (scheme 1, Fig. 1a) and triphenylphosphine hydrobromide (PPh3, HBr)38,40,41. The formation of benzofuran moity was achieved by an intramolecular reaction between ortho-hydroxybenzyltriphenylium salts h–n (Fig. 1) and appropriate benzoyl chlorides31,42–44. Hydrolysis of the methoxy groups of compound 1–14 was done by treatment with hydrogen iodide in acetic acid/acetic anhydride37,46, which resulted in corresponding hydroxy derivatives compounds 15–28 (scheme 2, Fig. 1b). The benzofuran structures were confirmed employing 1H NMR, 13C NMR and elemental analysis (see Supplementary Information)38–50.

Inhibitory activity of 2-phenylbenzofuran derivatives against AChE and BChE. To investigate the importance of hydroxyl substituents in the synthesized 2-phenylbenzofuran derivatives, we assessed the inhibitory effect of these compounds (15–28) on EeAChE and eqBChE activity by determining their inhibition potency IC50, which is concentration of inhibitor needed to reduce the enzyme activity by half. For the initial screening of the compounds, we used enzymes of non-human origin namely EeAChE and eqBChE due to their lower cost and high degree of similarity with their respective human enzymes.

The inhibition results of the compounds against the two enzymes are summarized in Table 1. We noted that compound 28, with three hydroxyl substituents in phenyl-ring and hydrogen atom in position 5 (R) and 7 (R1) of benzofuran scaffold did not exert any cholinesterase inhibitory activity. In general, except compounds 23 and
all other compounds displayed better activity against eqBChE enzyme. In detail, only six compounds (15, 17, 19–21 and 27) displayed inhibitory activity against eqBChE and with IC\textsubscript{50} values for EcAChE being equal or greater to 100 \textmu M. While, on the other hand the remaining compounds inhibited both the enzymes with varying efficiency. Among these derivatives, maximum inhibitory activity against eqBChE enzyme were displayed by compound 15 (IC\textsubscript{50} = 6.23 \textmu M) and 17 (IC\textsubscript{50} = 3.57 \textmu M), with two hydroxyl substituents in phenyl-ring and with presence of chlorine and bromine atoms respectively at position 7 (R1) of benzofuran scaffold. Interestingly, eqBChE inhibitory activity displayed by compounds 15 and 17 was about 4- and 8- times more active than the reference compound, galantamine (IC\textsubscript{50} = 28.3 \textmu M).

We therefore focused our attention on the compounds 15 and 17, which exhibited maximum inhibitory action against eqBChE enzyme. Further to evaluate the selective characteristics and type of inhibition, we investigated the kinetic behaviour of eqBChE at different concentration of \textit{S}-butyrylthiocholine iodide (BTCI) and inhibitors by Lineweaver-Burk plot analysis (Fig. 2).

Kinetic analysis of steady state inhibition data revealed that compound 15 acts as a mixed-type inhibitor. This is evident from Fig. 2, since increasing inhibitor concentration resulted in a family of straight lines with different slope and intercept. This behaviour furthermore suggested that compound 15 could bind not only with the free enzyme, but also with the enzyme–substrate complex. The equilibrium constants for binding with the free enzyme (K\textsubscript{1}) and with the enzyme–substrate complex (K\textsubscript{IS}) were obtained either from the slope or the 1/V\textsubscript{max} values (y-intercepts) plotted versus inhibitor concentration, respectively. The values of K\textsubscript{1} and K\textsubscript{IS} of compound 15 were determined to be 13.94 \textmu M and 8.66 \textmu M, respectively (Fig. 2b,c).

Instead, plots of the initial rates of eqBChE activity in the presence of increasing concentrations of compound 17 yielded a family of straight lines with different slopes that crossed the x-axis at similar points (Fig. 3). Thus, suggesting compound 17 as a non-competitive inhibitor. The inhibition constants K\textsubscript{1} and K\textsubscript{IS} for the compound 17 were determined to be 4.3 \textmu M and 4.7 \textmu M from the secondary plots (Fig. 3b,c). In a non-competitive inhibition, the inhibition constants (K\textsubscript{1}, K\textsubscript{IS}) have almost the same value.

The inhibitory activity of the most potent inhibitors (compounds 15, 17) was further investigated on hBChE enzyme; the results are presented in Table 2. We note that both these compounds inhibit hBChE enzyme with IC\textsubscript{50} values in the micromolar range and display similar IC\textsubscript{50} values.
However, with respect to eqBChE case, these compounds displayed a lower inhibitory activity against hBChE. Nevertheless, IC$_{50}$ values for the compounds obtained against hBChE enzyme is nearly 2 times lower to that obtained for the reference compound galantamine in the same assay conditions.

**Antioxidant activity assessment.** The antioxidant property of compounds 15–28 was evaluated by ABTS$^+$ assay and the results are represented as EC$_{50}$ values in Table 3.

We used Trolox as positive control to compare the antioxidant capacity of the subjected compounds. All the compounds were found to possess an ability to quench ABTS radical and displayed a scavenging activity better
or comparable to that of the positive control. Interestingly, compounds 15 and 17 that were also the most active BChE inhibitor, showed a good antioxidant activity with EC50 values of 14.9 µM and 16.7 µM, respectively.

Cytotoxicity assay analysis. After obtaining encouraging results from the inhibitory assay experiments, biosafety effectiveness of the two promising compounds (15 and 17) was further evaluated. Cells were treated with different concentration of each compound (0–100 µM) for 24 h and their potential cytotoxic effect on NSC-34 cells was determined by using MTT assay51. Viability of the cells treated with the compounds 15 and 17 and comparison to the control cells were performed (Fig. 4). Moreover, results also indicated that compounds 15 and 17 exhibited no considerable cytotoxic effect in NSC-34 cells at the concentration in which eqBChE activity was inhibited.

Table 2. Inhibition of hBChE by Compounds 15 and 17. hBChE inhibition is expressed as the mean ± SD (n = 3 experiments).

| Compound | IC50 (µM) ± SD |
|----------|----------------|
| 15       | 27.51 ± 1.82   |
| 17       | 27.46 ± 1.53   |
| Galantamine | 56.8 ± 4.11   |

Table 3. Antioxidant activity of compounds 15–28. aData represent the mean (± standard deviation, SD) of three independent experiments. bPositive control.
Molecular modeling studies. To predict how the compounds 15 and 17 bind to hBChE and to understand the molecular origin of their high inhibitory activity and selectivity, we performed molecular docking experiments. Docking results suggested similar interaction sites (Fig. 5a, b) and similar binding energy values (~7.5 kcal/mol), for the two compounds. The stability of the docking poses of the two compounds was investigated using MD simulations, which is a standard technique used to study the dynamical properties of biomolecules\textsuperscript{52–56}.

The stability of the systems during the MD simulations was evaluated by calculating the root mean square deviation (RMSD) of C-alpha atoms of protein residues (Fig. 5c) from the starting structure. The average RMSD values of protein bound compound simulations were lower than in free protein simulations, with lowest value noted for compound 17 complex simulations. Subsequently, the interaction energy between the hBChE residues and the two compounds was calculated by evaluating the non-bonded energy values comprising of Van der Waals and electrostatic energy in the two simulations. Both the complexes exhibited similar interaction energy values (Fig. 5d).

Figure 4. Effect of compound 15 and 17 on NSC-34 cell viability.

Figure 5. Molecular Modeling. (a) Superimposition of best-docked positions of compounds 17 (blue) and 15 (red) into binding site of hBChE protein. The protein is represented in cartoon representation, the active site residues in licorice, and loops leading to hBChE active site are shown. (b) Zoomed representation of hBChE interaction site for the two compounds, and key residues are shown. (c) RMSD plots for the free and compound-bound hBChE simulations. (d) Interaction energy plots between the compound and hBChE residues.
To understand the origin of this similarity, we carefully inspected the binding mode of the compounds in complex with hBChE using Ligplot\(^57\). The compounds (15 and 17) were stably bound to hBChE active site (Fig. 6) encompassing the region between peripheral anionic site (PAS) and the catalytic triad site (CAS). Figure 6 depicts five overlapping hBChE residues interacting with the two compounds. In detail, these residues are located in catalytic triad (S198), oxyanion hole (G117), acyl-pocket (L286, V288) and wall of BChE active site. The hydroxyl substituents in compound 17 interact with peripheral anionic site residue (Y332), while compound 15 interacts with oxyanion hole residue (G116) and residue T120.

To examine the effects of compound 15 and 17 on the protein structural dynamics, comparative analysis of a series of snapshots of the protein coordinates from MD simulations trajectories between the complex (bound to the compounds) and free protein was done. Calculation of all inter-residue cross-correlations fluctuations (see Methods) of C-alpha atoms resulted in a matrix of cross-correlation coefficient ($C_{ij}$) elements, which are displayed in a graphical representation as a dynamical cross-correlation map, shown in Fig. 7.

As expected, we note strong fluctuations occur along the diagonal occur (between the same residue), wherein $C_{ij}$ is always equal to 1. A clear difference in the cross-correlations maps between the free and complex simulations was observed (Fig. 7). With respect to free protein simulations (Fig. 7a), we observed between few domains, an increase in either a positive or a negative correlation dynamics for the complex simulations (Fig. 7b,c). In detail, the regions involved in higher negative correlated dynamics included residues 40–60, 170–190 and 380–500, while residues 230–280 displayed lower negative correlated dynamics. On the other hand, residues 430–470 exhibited higher positive correlated dynamics in the compound complexes. As expected, most of these regions are in close vicinity to the hBChE active site gorge. Interestingly, only for compound 17 complex (Fig. 7b), positive correlation dynamics was noted between the domains surrounding the BChE active site gorge, i.e. residues 240–280 and 300–330, respectively.

**Discussion**

There is increasing clinical evidence suggesting an important role of BChE in the regulation of ACh levels and in particular in the development and progression of AD. Particularly, in progressed or late stage of AD, BChE mostly dominates hydrolysis of ACh\(^58\). Moreover, alongside its involvement in AD progression, an emerging role of BChE as a prognostic marker (which determines the progress of the disease) in liver and non-liver diseases, as well as in protein-energy malnutrition and obesity, has been reported\(^15,59\). Design and development of compounds with the ability to selectively inhibit BChE would not only improve understanding of the aetiology of AD but also assist in developing wider variety of new treatments. Therefore, the objective of our study has been to design and develop 2-phenylbenzofuran compounds that display selective BChE inhibitory activity employing biochemical, kinetics and computational techniques.

In our recent study\(^34\), we reported that the contemporary presence of a hydroxyl group in the para position of the 2-phenyl ring and a halogen substitution at position 7 (R\(^7\)) of the benzofuran scaffold resulted in a good and selective BChE inhibition, with best inhibitor displaying an IC\(_{50}\) of 30 µM. Following the results of our previous findings, in this present work we decided to explore the importance of the number and position of hydroxyl groups located in the 2-phenyl ring of the benzofuran moiety. We therefore synthesized new 2-phenylbenzofuran compounds with two hydroxyl substituents (compounds 15–21) and with three hydroxyl substituents (compounds 22–28). Galantamine was used as our reference compound. The inhibitory action of the newly synthesized compounds presented in Table 1 demonstrate that, regardless the type of substituent at position 7 of benzofuran scaffold, the 2-phenylbenzofuran derivatives with two hydroxyl substituents (compounds
In this study, a series of hydroxylated 2-phenylbenzofurans compounds were designed, synthesized and their selective inhibitory activity BChE was evaluated. Combining biochemical analysis and computational approaches, we identified two potent BChE inhibitors as compound 17 (IC₅₀ = 3.5 μM) and compound 15 (IC₅₀ = 6.25 μM), with the presence of two hydroxyl substituents in meta position of the 2-phenyl ring and bromine or chlorine at position 7 of benzofuran moiety. The BChE selective inhibition property decreased with the introduction of a third hydroxyl group in the 2-phenyl ring of the compounds. Detailed kinetic experiments revealed compound 15 as a mixed-type inhibitor, while 17 as non-competitive inhibitor of BChE activity. Experimental results were confirmed by MD simulations, which revealed a conserved interaction pattern resulting in similar interaction energy values. Finally, compounds 15 and 17 examined on hBChE revealed 2-times more active inhibitory action...
than the reference compound. In conclusion, gathering the information obtained in this study, compounds 15 and 17 could be considered as promising candidates for the design and development of drugs against AD.

Methods

Chemistry. Starting materials and reagents were obtained from commercial suppliers (Sigma-Aldrich) and were used without further purification. Melting points (mp) are uncorrected and were determined with a Reichert Kofer thermopan or in capillary tubes in a Büchi 510 apparatus. 1H NMR and 13C NMR spectra were recorded with a Varian INOVA 500 spectrometer using [D$_6$]DMSO or CDCl$_3$ as solvent. Chemical shifts (€) are expressed in parts per million (ppm) using TMS as an internal standard. Coupling constants (J) are expressed in hertz (Hz). Spin multiplicities are given as s (singlet), d (doublet), dd (doublet of doublets), and m (multiplet). Elemental analyses were performed by using a Perkin Elmer 240B microanalyzer and are within 0.4% of calculated values in all cases. The analytical results indicate 98% purity for all compounds. Flash chromatography (FC) was performed on silica gel (Merck 60, 230–400 mesh); analytical TLC was performed on pre-coated silica gel plates (Merck 60 F254). Organic solutions were dried over anhydrous Na$_2$SO$_4$. Concentration and evaporation of the solvent after reaction or extraction was carried out on a rotary evaporator (Büchi Rotavapor) operating under reduced pressure.

Preparation of Methoxylathed 2-phenylbenzofuran. A mixture of 2-hydroxybenzyltriphenylphosphonium bromide (0.50 g, 1.11 mmol) and benzoyl chloride (0.12 mL, 1.11 mmol) in a mixed solvent (toluene 20 mL and Et$_3$N 0.5 mL) was stirred under reflux for 2 h. The precipitate was removed by filtration. The filtrate was concentrated, and the residue was purified by silica gel chromatography (hexane/EtOAc 9:1) to give the desired compounds 1-14,41,42.

Preparation of Hydroxylated 2-phenylbenzofurans. A solution of the corresponding methoxy-2-phenylbenzofuran (0.11 g, 0.50 mmol) in acetic acid (5.0 mL) and acetic anhydride (5.0 mL), at 0°C, was prepared. Hydriodic acid 57% (10.0 mL) was added drop-wise. The mixture was stirred under reflux temperature for 3 h. The solvent was evaporated under vacuum and the dry residue was purified by FC (dichloromethane/methanol 9:80:2) to give the desired compound 15-28.

Cholinesterase assay. The enzymes and reagents for biochemical assays were obtained from Sigma-Aldrich. Kinetic assays of cholinesterase activity were performed using Ellman’s method and analyzed as previously described. Briefly, in the microplate assay the reaction mixture contained phosphatase buffer (0.1 M, pH 8.0), AChE or BChE solution (0.3 or 0.15 U/mL respectively), 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB; 1.5 mM), and inhibitor dissolved in 1% DMSO at the desired concentrations or DMSO alone (control) in a final volume of 0.2 mL. Finally, acetylthiocholine iodide (ATCI) or S-butyrylthiocholine iodide (BTCl) (1.5 mM) as the substrate was added to the reaction mixture and the absorbance immediately monitored at 405 nm. The activity of the enzymes was performed at 25 °C.

Acetylcholinesterase was from Electrophorus electricus (EeAChE), while butyrylcholinesterase was from equine serum (eBChE) or human serum (hBChE). Each inhibitor was evaluated at six concentrations (ranging from 0.5 to 100 μM). Galantamine was used as the standard cholinesterase inhibitor.

The inhibition potency was expressed in IC$_{50}$ values, which represent the inhibitor concentration giving 50% inhibition of cholinesterase activity. IC$_{50}$ values were calculated by the interpolation of dose-response curves using GraphPad Prism 6 (Graphpad Software, San Diego, California, USA). IC$_{50}$ values displayed represent the mean ± standard deviation for three independent assays.

Kinetic characterization was performed by constructing Lineweaver-Burk plots by plotting 1/ arousal vs 1/[S] in the presence of different concentrations of inhibitor and substrate. Kinetics constants were determined by the replots of the slopes (K$_m$/V$_{max}$) or 1/V$_{max}$ versus the inhibitor concentration.

Antioxidant activity. Total free radical-scavenging capacity of the compounds was determined by ABTS$^+$ [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] method using Trolox as antioxidant standard, as previously reported. Briefly, the free radical ABTS$^+$ was produced by reacting 7 mM ABTS with 2.45 mM potassium persulfate in aqueous solution and kept in the dark for 24 h at room temperature before use. After appropriate dilution, each compound (10 μL) was added to 1 ml of ABTS$^+$ solution and the absorbance at 734 nm was recorded after 1 min incubation. Results were expressed as EC$_{50}$ values (μM), the concentration of sample necessary to give a 50% reduction in the original absorbance.

Cell viability. Mouse motor neuron like cell line (NSC-34) was purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, NY, USA), and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO$_2$. Cell viability was detected by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This is a colorimetric assay for measuring the activity of mitochondrial enzymes in living cells that convert MTT into purple formazan crystals. Briefly, cells were seeded in a 96-well plate (10$^4$ cells/well) and incubated with samples at concentration ranging from 10 to 100 μM for 48 h. As DMSO was used as solvent for compounds, all activities were performed also in the presence of DMSO alone, as solvent control. After incubation time, cells were labelled with MTT solution for 3 h at 37°C. The resulting violet formazan precipitates were dissolved in isopropanol and the absorbance of each well was determined at 590 nm using a microplate reader with a 630 nm reference.

Molecular Modeling. High-resolution three-dimensional protein structure of hBChE was obtained from protein data bank (PDB id: 4TPK). For the compounds (15 and 17), the three-dimensional coordinates were
generated using Open Babel software. The geometry of the compounds were optimized using the Hartree-Fock basis set 6-31 G* within Gaussian03 software package. The charges and the force field parameters of the compounds were evaluated following the standard protocol within AMBER software tools.

Molecular docking of the compounds into hBChE protein was performed using SwissDock web server, which is based on the docking software EADock DSS. The docking poses of the compounds were accurately chosen with a blind docking procedure that considers the entire protein surface as a potential target. Using this procedure, a large number of ligand binding modes (~15000) were generated, with the simultaneous rough interaction energy estimation. The binding modes possessing favorable energies were then ranked and classified into different clusters, this time based on the full fitness scoring function. The most consistent and favorable conformation chosen from 10 independent docking runs for each compound was further considered for MD simulations.

The hBChE–compound complexes were built using leap module of Amber11. Each complex was inserted separately in an explicit water-box with a minimum distance of 1.8 nm between the solute and box boundary. Further details about the simulation box size and the total number of atoms for each complex are provided in Supplementary Table S1. We used amber force-field parameters for hBChE protein and TIP3P parameters for water molecules. Energy minimization, followed by heating of the complexes to temperature 300 K, was done with positional restraints on C-alpha atoms. The positional restraints were gradually removed during the simulation time and an equilibration run of 10 ns was performed. The time step used in MD simulation was of 2 fs using SHAKE algorithm. Simulations were performed in NPT ensemble using periodic boundary conditions. All-atom MD simulations of free protein and protein–compound complexes were performed for a simulation time of 100 ns employing NAMD software package.

The stability of systems was evaluated by calculating the RMSD values for the C-alpha atoms of residues during MD simulations, using VMD. The interaction energy between the compound and protein residues was calculated by evaluating the non-bonded energy values comprising of Van der Waals and electrostatic energy, using the energy plugin of NAMD software. A cut-off distance of 12 Å was used for non-bonded interactions and for the electrostatic interaction we also adopted the particle mesh Ewald scheme. The dynamic cross-correlation coefficients for C-alpha atoms was calculated on 1000 snapshots extracted from 100 ns MD trajectories using Prody software. The matrix of all inter-atomic cross-correlations of atomic fluctuations $C_{ij}$ where $i$ and $j$ are C-alpha atoms, can be represented as a dynamical cross-correlation map. If the fluctuations of two C-alpha atoms are completely correlated then $C_{ij} = 1$ (red), if anticorrelated then $C_{ij} = -1$ (blue), and if $C_{ij} = 0$ (white) then the fluctuations of $i$ and $j$ are not correlated.

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Author Contributions
A.K., E.P., G.L.D. and A.F. conceived and designed the experiments. A.K. performed M.D. simulations. A.K., E.P. performed biochemical assays. P.C. was involved in cell viability assay. M.J.M., D.V. and G.L.D. were involved in synthesis of benzofurans and F.D. analyzed and discussed computational results. F.P., A.D.P., R.M., B.E. and A.F. performed biochemical experiments. A.K., F.P., G.L.D. and A.F. conceived and designed the experiments. A.K. performed M.D. simulations. A.K., E.P.

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