Inhibition of acetylcholinesterase and butyrylcholinesterase with uracil derivatives: kinetic and computational studies

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ABSTRACT

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE) inhibitors are interesting compounds for different therapeutic applications, among which Alzheimer’s disease. Here, we investigated the inhibition of these cholinesterases with uracil derivatives. The mechanism of inhibition of these enzymes was observed to be due to obstruction of the active site entrance by the inhibitors scaffold. Molecular docking and molecular dynamics (MD) simulations demonstrated the possible key interactions between the studied ligands and amino acid residues at different regions of the active sites of AChE and BuChE. Being diverse of the classical AChE and BuChE inhibitors, the investigated uracil derivatives may be used as lead molecules for designing new therapeutically effective enzyme inhibitors.

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1. Introduction

Alzheimer’s disease (AD) is defined as a neurodegenerative condition characterised by abnormal behaviour, intellectual reduction, being a major public health problem, especially due to the increasing elderly population in developed countries. In spite of the fact that AD pathogenesis has not been clarified as yet, one of the most important theories was the “cholinergic hypothesis”. A defect in the levels of acetylcholine (ACh) and butyrylcholine (BCh) acting as neurotransmitters was observed in the brains of patients with AD. The inhibition of AChE and BuChE enzymes that hydrolyse ACh and BCh neurotransmitters has become thus a treatment option of AD. For this reason, many research groups have conducted investigations of the inhibitory activity for these enzymes involved in AD pathogenesis. AChE catalyses the hydrolysis of ACh, which has an important role in cognition and memory. The observation of ACh depletion in AD patients due to the loss of cholinergic neurons constitutes a strategy for their treatment. Drugs such as tacrine, donepezil, galantamine, and rivastigmine are AChE enzyme inhibitors, mainly increasing the amount of ACh by blocking ACh hydrolysis. While this strategy works in about half of the patients for several years, curative therapy continues to be an unachieved goal. These drugs interact with the active site of the AChE: tacrine, without altering the structure of the enzyme (being a reversible inhibitor), whereas rivastigmine changing it: the carboxamoyl group of rivastigmine was found covalently bound to AChE, with the rest of the drug in the catalytic site and with its phenol functional group exposed to the solvent.
5-Fluorouracil (5-FU) is an uracil analogue used as an antineoplastic drug (antimetabolite). 5-FU interferes with DNA synthesis by blocking DNA polymerase and thymidylate synthetase enzymes. 5-FU and its metabolites have several different mechanisms of action. In vivo, 5-FU is converted to the active metabolite 5-fluorouridyl monophosphate (FUMP); replacing U, FUMP incorporates into RNA and inhibits RNA processing, thereby inhibiting cell growth. Fluorouridyl is used for treating malignant neoplasms of the liver and gastrointestinal tract and hepatic metastases. Sorivudine is a uridine derivative with potent antiviral activity against herpes simplex and varicella zoster viruses. Sorivudine acts by inhibiting DNA polymerase by converting it into triphosphate form in cells. Uramustine, a uracil derivative, is an alkylating antineoplastic agent used in lymphatic malignancies that causes mainly gastrointestinal and bone marrow damage. In this study, the in vitro inhibition properties and in silico calculations of these uracil derivatives 2–9 in their interaction with AChE and BuChE were investigated.

2. Materials and methods

2.1. Chemistry

1-Acetyl-1H-pyrimidine-2,4-dione (2), 5-bromo-1H-pyrimidine-2,4-dione (3), 5-Bromo-1-(toluene-4-sulfonyl)-1H-pyrimidine-2,4-dione (4), 5-Bromo-1-methanesulfonfyl-1H-pyrimidine-2,4-dione (5). Uracil derivatives 2–5 were synthesised according to ref 11. 5-Fluorouracil (6), 6-methyluracil (7), 1,3-Dimethyluracil (8), 5-Hydroxymethyluracil (9), and other chemicals were obtained commercially from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

2.2. Biological activities

The AChE and BuChE enzymes inhibitory activities with the target uracil derivative 2–9 were determined by using the Ellman method. Neostigmine was used as the reference drug in this study. The IC50 values obtained for compounds 2–9 are summarised in Table 1.

1 mg of each inhibitor was dissolved in 1 mL DMSO and then diluted to various concentrations with deionised water. To determine the cholinesterase inhibition activity, six serial dilutions of the inhibitors were measured. The reaction system was composed of 5–60 µL inhibitor sample, 200 µL buffer (1 M, pH 8.0: Tris-HCl buffer for the AChE assay and phosphate buffer for the BuChE assay), 50 µL DTNB (0.5 mM), 50 µL acetylthiocholine iodide/S-butrylthiocholine chloride (10 mM), and 10 µL enzyme (0.28 units mL for the AChE assay and 0.32 units/mL for the BuChE assay). The reaction was initiated upon addition of the enzyme. The reaction system was prepared at room temperature in a quartz cuvette. The blank reading was composed of all chemicals except the inhibitor.

The absorbance of the reaction mixture was measured at 412 nm within 5 min from the start of the reaction on a Thermo Scientific Evolution 200 Series (UV-VIS) spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). The absorbance for each reaction mixture was measured three times within 5 min of adding the enzyme and the results are reported as mean ± standard deviation. The inhibition properties are reported as IC50 values which were determined graphically from inhibition curves of log inhibitor concentration vs. percent of inhibition. IC50 values represent the concentration of inhibitor required for 50% inhibition of the enzyme.

2.3. In silico studies

2.3.1. Ligand and protein preparation

Maestro Molecular Modeling Package was used for protein and ligand preparations. First, AChE (PDB ID: 4EY7) and BuChE (PDB ID: 5DYW) crystal structures were retrieved from the protein data bank, then AChE and BuChE amino acid sequences were downloaded in UniProt to crosscheck and fix the unresolved residues in the crystal structures. Crosslink Proteins tool in Maestro was utilised to fill the missing amino acid residues in implicit solvent environment. “A” chain of each crystal structures was used for further steps. The missing elements in the proteins (e.g. hydrogen atoms and missing atoms) were added by Protein Preparation Wizard module. Water molecules near 5 Å of the ligands were kept and other water molecules were removed. The pKa prediction and protonation state of ligands was predicted at pH 7. PROPKA was used to assign the protonation states of the protein residues at pH 7. Subsequently, restrained minimisation (with 0.30 Å RMSD heavy atom convergence) was realised for the systems with OPLS force field. Ligands were drawn with 3D Builder tool and subsequently Ioniser module in conjunction with LigPrep tool of Maestro molecular modelling suite was used for compound preparation and energy optimisation with OPLS force field.

| Ligand                | AChE IC50 (µM) | AChE Docking score | BuChE IC50 (µM) | BuChE Docking score |
|-----------------------|---------------|--------------------|-----------------|---------------------|
| Neostigmine           | 0.136         | 59.92              | 0.270           | 44.41               |
| Donepezil             | NA            | 114.72             | NA              | 72.38               |

Table 1. IC50 values obtained from AChE and BuChE (µM). Docking scores of corresponding calculations are also shown in the table.
2.3.2. Ligand docking

(i) GoldScore scoring function implemented in GOLD (Genetic Optimization for Ligand Docking, v.5.3) docking programme\textsuperscript{27} was used in order to obtain the predicted binding poses for protein-ligand complexes and binding energies of the studied ligands towards AChE and BuChE proteins. Protein binding sites of AChE and BuChE targets were defined according to their co-crystallised ligands allowing to cover the whole ligand binding cavity regions during the docking simulations. 50 poses were generated for each ligand where protein residues were treated as rigid bodies and ligands were treated flexible. Water molecules were set in toggle and spin states at the surrounding ligand sites. Search efficiency was set to 100\% while 10,000 and 125,000 minimum and maximum operation values were selected, respectively. Early termination was turned off and diverse solution generation selection was invoked.

(ii) In addition, Glide/SP and Glide/XP docking algorithms in Maestro were also used for flexible ligand docking simulations\textsuperscript{28–31}. Protein grid generation calculation steps (prior to docking) and both standard (SP) and extra precision (XP) docking settings were used with default values. Docking simulation boxes were defined from the centroids of their crystal ligand binding sites and maximum 50 poses were requested for each ligand.

2.3.3. MD simulations

The top-docking scored poses of molecule 4 complexed with AChE and BuChE were used in the MD simulations. The buffer size of the system box was set to $10 \times 10 \times 10 \, \text{Å}^3$, and the box shape was specified as orthorhombic. Explicit water molecules (SPC) were used in the preparation of the system, and also 0.15 M NaCl ion concentrations were added to it for the neutralisation of the system. In MD simulations, NPT ensemble at 310 K with Nosé-Hoover temperature coupling and at constant pressure of 1.01 bar via Martyna-Tobias-Klein pressure coupling was provided\textsuperscript{26}. All the systems were prepared and put through the MD simulations by using Desmond programme employing the OPLS2005 force field and RESPA integrator\textsuperscript{28}. There were no constraints on the generated systems and the initial velocity values are used as default. The prepared system was subjected to 100 ns of MD simulations run.

3. Results and discussion

In an earlier report from our group, the inhibitory ability of compounds 2–9 on human carbonic anhydrase (hCA) was investigated\textsuperscript{15}. Some of these uracil derivatives demonstrated good to moderate inhibition profiles against hCA I and hCA II\textsuperscript{14,32}. Inhibitors of carbonic anhydrase (CA) have been carried out in many therapeutic applications, especially antiglaucoma activity. It was thus decided to screen them against AChE and BuChE. AChE and BChE inhibitors are used in the treatment of many neurodegenerative diseases, especially Alzheimer’s disease\textsuperscript{8–11}. Compounds 2–9 (Figure 1), possessing different functional groups on the pyrimidine scaffold, were evaluated for their inhibitory activity of AChE and BuChE by means of the Ellman’s colorimetric assay\textsuperscript{15}. Neostigmine, commercially available cholinesterase inhibitor was used as the reference compound.
The concentration of the uracil derivatives (inhibitors 2–9) required to inhibit 50% of AChE and BuChE activity was calculated from various inhibitor concentrations and reported in Table 1. A comparison of the IC50 values of 2–9 indicated that their inhibition was mixed in nature, IC50 values of the inhibitors ranged from 0.088 to 0.388 μM for AChE and from 0.137 to 0.544 μM for BuChE. The results demonstrated that the compounds showed IC50 values weaker compared to neostigmine (IC50 AChE = 0.136 μM and IC50 BuChE = 0.084 μM) against both AChE and BuChE. The strongest inhibition was observed with 4 (IC50 = 0.088 μM) against AChE but was 1.54-fold active compared to neostigmine. Compound 4 (IC50 = 0.137 μM) exhibited the strongest inhibition of BuChE; however, 1.63-fold less active compared to neostigmine. Thus, a computational study was performed in order to rationalize the observed inhibitory activities. Compounds 2–9 docking scores ranged from −5.06 to −7.90 kcal/mol for AChE and −5.93 to −7.97 kcal/mol for BuChE (Glide/XP results).

Both GOLD and Glide docking results fit to experimental findings (Table 1). Scores of top docking poses of compound 4 show higher scores compared to other molecules. In GOLD, higher GoldScore Fitness scoring values represent tighter binding interactions. Results also show ligand efficiency scores (LIE) of studied molecules. In order to escape the affinity-biased selection and optimisation towards larger ligands, Hopkins et al.33 recommended to assess binding affinity in relation to number of heavy atoms in a molecule and introduced the term ligand efficiency (the average affinity contribution per atom is considered) instead of considering the affinity of the whole compound. This provides a way to compare the affinity of molecules corrected for their size. In our case, we used Glide/XP scores for the calculation of ligand efficiency scores (ligand efficiency: GlideScore/number of heavy atoms). Results show that compound 3 has top-scored LIE values both in AChE and BuChE.

Figure 2 represents the 2D and 3D ligand interaction diagrams of top-docking poses of compound 4 as well as a well-known AChE inhibitor donepezil at the binding pocket of the target. Both molecules interact common active site residues at the AChE; such as Phe295, Trp86, and Trp286. However, as compared to top-poses of Glide SP and Glide XP, top docking pose of GoldScore has an alternative orientations at the binding pocket. The Br-uracil fragment locates between the Ser125 and Glu202 residues where this orientation allows compound 4 to make hydrogen bonding interaction within Glu202 and π–π stacking interaction with Trp86 (Figure 2). For BuChE, both three docking algorithms predict identical binding orientation of compound 4 (Figure 3) where Trp82 and Tyr 332 form π–π stacking interaction with the aromatic rings of the ligand while Glu197 and His438 residues make hydrogen bonding interactions with the Br-uracil fragment of the molecule.

In order to investigate the structural and dynamical profiles of molecule 4 at the binding pockets of AChE and BuChE, MD simulations were performed for the top-docked poses attained from Glide/XP and GoldScore for AChE and Glide/XP for BuChE, using Desmond. Figures 4 and 5 show a timeline representation of the interactions and contacts (H-bonds, hydrophobic, ionic, water bridges) of compound 4 at the binding pockets of AChE and BuChE. The top panel shows the total number of specific contacts the AChE makes with the molecule 4 over the course of the trajectory. The bottom panel represents which residues interact with the ligand 4 in each trajectory frame. Some residues make more than one specific contact with the ligand, which is represented by a darker shade of orange, according to the scale to the right of the plot. Mostly observed contacts at AChE are from Trp286, Phe295, Arg296, Phe338, and Tyr341. Corresponding interactions were Trp82, Glu197, Tyr332, His438, and Tyr441 at the BuChE. Interactions that occur more than 30.0% throughout the simulation are also shown Figures 4 and 5. The ligand torsions plot summarises the conformational evolution of every rotatable bond in the ligand 4 throughout the simulation (Figure 6). The top panel
Figure 3. Superposition of top-docking poses of compound 4 at BuChE binding site, generated by Glide/SP (wheat), Glide/XP (blue), and GoldScore (pink).

Figure 4. Timeline representation of the interactions and contacts throughout the MD simulations of 4 at the binding pocket of AChE. Protein-ligand interactions are monitored throughout the MD simulations. These interactions are categorised into four types: Hydrogen Bonds, Hydrophobic, Ionic, and Water Bridge. Interactions that occur more than 30.0% of the simulation time in the selected trajectory are shown in 2D interaction diagram.

shows the 2D schematic of a ligand with colour-coded rotatable bonds. Each rotatable bond torsion is accompanied by a dial plot and bar plots of the same colour. The radial plots describe the conformational change of the torsion throughout the MD simulations. The beginning of the simulation is in the centre of the radial plot and the time evolution is plotted radially outwards. The bar plots summarise the data on the dial plots, by showing the probability density of the torsion. Results show that rotatable bonds are quite stable throughout the simulations. The histogram plot and torsional analysis of ligand give detailed information into the conformational change of the ligand 4 at the binding sites of AChE and BuChE.

AChe and BuChE are enzymes which play an important role in memory and cognition. They catalyse the hydrolysis of acetylcholine causing a loss of communication between nerve cells. This leads to a loss of brain function and causes AD. Treatment of AD relies on the restoration of the level of acetylcholine. Pharmaceutical research has thus been focusing on cholinesterase inhibitors as treatments for cognitive disorders. Commercially available medicines for AD suffer from drawbacks such as gastrointestinal upset and bioavailability problems and therefore new cholinesterase inhibitors are continuously being investigated. We thus screened uracil derivatives 2–9 for their inhibitory activity.
Uracil derivative 8 (IC\textsubscript{50} = 0.388 μM) showed the least potent inhibitory activity against AChE. Decreasing the number of methyl groups on the aromatic ring showed an improvement of the IC\textsubscript{50} values obtained, 7 (0.191 μM) with methyl group demonstrated a 2.03-fold decrease of inhibition activity while 9 (0.205 μM) with hydroxymethyl group showed a 1.89-fold decrease of inhibition activity compared to compound 8. However, compound 4 (0.088 μM) possessing 1-(toluene-4-sulfonyl) group showed better inhibitory activity compared to other seven uracils. The difference between the other tested uracils and 4 is that this molecule is a more voluminous derivative. The stronger inhibition capability of uracil derivative 4 may suggest that the compound’s geometry is more suitable for enzyme interaction when (toluene-4-sulfonyl) group is N1. These results may indicate that the substituent position is more important for inhibition activity compared to toluene-4-sulfonyl groups present in the molecule. A more in-depth study will be done to investigate this theory. To determine the importance of the toluene-4-sulfonyl group on inhibitory activity, other seven uracils were compared to compound 8. Adding a mesylate group (4) to toluene-4-sulfonyl group (5) the inhibitory activity decreased 1.26-fold, suggesting that the toluene-4-sulfonyl moiety is an important functional group for enzyme activity. Compound 4 exhibited a 1.71-fold stronger inhibitory profile compared to uracil 3.

In the case of BuChE, uracil derivative 4 showed the most promising activity with an IC\textsubscript{50} value of 0.137 μM. This is in agreement with the results observed for N1 position toluene-4-sulfonyl group substituted uracil. 3 (IC\textsubscript{50} = 0.292 μM) is a slightly weaker
inhibitor (2.13-fold) compared to uracil derivative 4 but possessed better inhibitory potential compared to the other compounds tested (see entry 6–9). 4 was a slightly better inhibitor (1.42-fold) compared to 5 (0.195 μM), this once again supports the N1 toluene-4-sulfonyl substituted 5 Br-uracil theory as discussed before. The weakest inhibitor amongst the set of compounds was 8 with an IC\textsubscript{50} value of 0.544 μM. Tested uracil derivatives (2–9) showed similar results for both AChE and BuChE.

4. Conclusions

As discussed, the screening led to interesting results and can help with the development of more effective drugs to slow down or stop AD. We will expand the study to explore the structure activity relationship of uracil derivatives 2–9, in addition, a comparison of these uracil derivatives with other aromatic compounds will be investigated. These compounds could also be used as precursors or building blocks in the preparation of much more effective drug molecules.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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