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Docking-based Design of Galantamine Derivatives with Dual-site Binding to Acetylcholinesterase

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Abstract: The enzyme acetylcholinesterase is a key target in the treatment of Alzheimer's disease because of its ability to hydrolyze acetylcholine via the catalytic binding site and to accelerate the aggregation of amyloid-β peptide via the peripheral anionic site (PAS). Using docking-based predictions, in the present study we design 20 novel galantamine derivatives with alkylamide spacers of different length ending with aromatic fragments. The galantamine moiety blocks the catalytic site, while the terminal aromatic fragments bind in PAS. The best predicted compounds are synthesized and tested for acetylcholinesterase inhibitory activity. The experimental results confirm the predictions and show that the heptylamide spacer is of optimal length to bridge the galantamine moiety bound in the catalytic site and the aromatic fragments interacting with PAS. Among the tested terminal aromatic fragments, the phenethyl substituent is the most suitable for binding in PAS.

Keywords: acetylcholinesterase inhibitors · galantamine · molecular docking · ChemPLP · dual-site binding · amyloid beta peptide

1 Introduction

The enzyme acetylcholinesterase (AChE) is a key target in the treatment of Alzheimer's disease (AD). It hydrolyses acetylcholine (ACh), a neurotransmitter playing a main role in memory and cognition. The AChE inhibition enhances the levels of ACh and improves the cholinergic transmission impaired in AD.[1] Additionally, AChE accelerates the aggregation of amyloid-β peptide (Aβ) and forms stable neurotoxic complexes with Aβ.[2–4] The complexes induce Aβ-dependent deregulation of intracellular Ca^{2+} in hippocampal neurons, mitochondrial dysfunction, neurite network dystrophia and apoptosis.[4]

AChE has several binding domains (sites): catalytic, anionic, acylic, oxyanionic and peripheral anionic.[5–9] The catalytic triad consists of three residues – Ser203, Glu334 and His447 (*h*AChE) – and is situated at the bottom of 20 Å deep and narrow binding gorge. The anionic domain binds the quaternary trimethylammonium choline moiety of ACh and is built by four aromatic residues: Trp86, Tyr130, Tyr337 and Phe338. Trp86 is involved in cation-π interactions with the positively charged head of ACh. The acyl pocket determines the selective binding of ACh. It consists of two bulky residues – Phe295 and Phe297 – which prevent the access of larger choline esters. Gly121, Gly122 and Ala204 form the oxyanion hole which hosts one molecule of structural water. This molecule forms a dense hydrogen-bond network between enzyme and substrate and stabilizes the substrate tetrahedral transition state. The peripheral anionic site (PAS) lies at the entrance to binding gorge and consists of five residues: Tyr72, Asp74, Tyr124, Trp286 and Tyr341. After the catalytic site, PAS is the second most important drug target binding site of AChE. It modulates the catalysis allosterically[10] and is implicated in non-cholinergic functions as amyloid deposition,[11] cell adhesion and neurite outgrowth.[12,13] The Aβ peptide binds close to PAS and the blockade of this domain prevents the AChE-induced Aβ aggregation.[11]

The multiple binding site attacks to AChE was initiated by the development of bis(7)-tacrine analogs – acetylcholinesterase inhibitors (AChEIs) designed to bind at both catalytic site and PAS.[14] This finding prompted the development of several series of dual- or multiple-site binding ligands with anti-AD activities.[15–23] Molecules with different but synergistic mechanisms of action have been combined in hybrid entities.[24–34] The multi-target design strategies applied for the treatment of AD have been extensively reviewed recently.[35–38]

Galantamine (GAL) is an AChE inhibitor widely prescribed as an anti-AD drug.[39–41] Additionally, GAL is an allosteric

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modulator of nicotinic acetylcholine receptors (nAChRs).[42–45] The stimulation of nAChRs increases the intracellular Ca^{2+} levels and facilitates noradrenaline release; both effects enhance the cognitive brain function.[46] The treatment of rat microglia with GAL significantly enhanced microglial Aβ phagocytosis and facilitated Aβ clearance in brains of rodent AD models.[47] It is evident that GAL is a multiple target drug and is a good scaffold for further development. The rational design here is focused on the elongation of N-side chain ending by a bulky substituent able to interact with PAS. Several series of GAL derivatives with dual-site binding to the enzyme have been prepared and tested.[48–52] All of them showed good AChE inhibitory activities.

Molecular docking is a widely used structure-based method for virtual screening and drug design, used solely or in combination with 2D- and 3D-QSAR, high-throughput screening and/or machine learning methods.[53–65] Recently, we designed several galantamine derivatives with indole moiety in the side chain and predicted their AChE binding affinities by molecular docking simulations.[52] The four best predicted compounds were synthesized and tested. All of them showed affinities between 11 and 95 times higher than the affinity of GAL. The novel derivatives had dual-site binding to the enzyme – the GAL moiety binds to the catalytic site and the indole moiety interacts with PAS.

In the present study, we design 20 novel GAL derivatives with alkylamide spacers of different length ending with aromatic fragments. The aromatic fragments are selected as suitable for binding to the aromatic residues in PAS because of their ability to take part in hydrophobic and π-π interactions. The designed ligands are docked in *rh*AChE and the best scored compounds are synthesized and tested. Two of them show anti-AChE inhibitory activity in the nano- and subnanomolar range.

2 Materials and Methods

2.1 Molecular Docking Protocol

The X-ray structure of *rh*AChE in complex with GAL (pdb id: 4EY6, R = 2.15 Å)[66] was used as a template for docking calculations by GOLD v. 5.1.[67] The molecular docking protocol was optimized previously in several steps using two training sets.[52,68] At each step the correlation between the docking scores and pIC_{50} ($-\log IC_{50}$) values of the training compounds was evaluated. The highest correlation was achieved at the following settings: scoring function ChemPLP,[69] flexible binding site, flexible ligand, radius of the binding site 10 Å and one water molecule (HOH846) kept within the binding site (toggle on and spin). According to the X-ray structure of the complex GAL – rhAChE,[66] only one water molecule was left in the binding gorge, making a hydrogen bond network between GAL, Ser203, Gly121 and Gly122. Ten amino acids from the binding site situated in a close proximity to the binding ligands were selected as flexible. These were Tyr72, Asp74, Trp86, Tyr124, Ser125, Trp286, Phe297, Tyr337, Phe338 and Tyr341. Each run generated 10 docking poses. The poses were ranked by two criteria: 1) highest fitness score and 2) rmsd (root mean square deviation) lower than 1.5 Å. Each docking run was repeated three times. The average score of the highest scoring poses was considered.

The molecular mass, logP, number of hydrogen bond donors and acceptors in the molecules were calculated by ACD/logD v.9.08 (Advanced Chemistry Development, Inc.). The BBB permeability was predicted by the BBB Predictor (http://www.cbligand.org/BBB/). It classifies whether a compound can cross the blood-brain barrier (BBB+) or not (BBB-). This predictor was built by applying SVM and LiCABEDS[70] algorithms on four types of fingerprints (MACCS, OpenBabel FP2, Molprint and PubChem) of 1593 reported compounds.[71]

2.2 Synthesis

2.2.1 General

Reagents were commercial grade and used without further purification. Thin layer chromatography (TLC) was performed on aluminum sheets pre-coated with Merck Kieselgel 60 F_{254} 0.25 mm (Merck). Flash column chromatography was carried out using Silica Gel 60 230–400 mesh (Fluka). Commercially available solvents for reactions, TLC and column chromatography were used after distillation (and were dried when needed). Melting points were determined in a capillary tube on SRS MPA100 OptiMelt (Sunnyvale, CA, USA) automated melting point system (uncorrected). Optical rotation ($[\alpha]_D^{20}$) were measured on JASCO P-2010 polarimeter. The NMR spectra were recorded on a Bruker Avance II+ 600 (600.13 for ^{1}H MHz and 150.92 MHz for ^{13}C NMR) spectrometer with TMS as internal standards for chemical shifts (δ, ppm). ^{1}H and ^{13}C NMR data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants (Hz), integration, identification. The assignment of the ^{1}H and ^{13}C NMR spectra was made on the basis of DEPT, COSY and HSQC experiments. The ^{1}H and ^{13}C NMR spectra of the synthesized compounds are given as Supplementary data. LC–MS analyses were performed using a Q Exactive Plus Orbitrap Mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), equipped with an electrospray (ESI) probe. The spectra were recorded on a positive mode using a MS Full Scan mode.

2.2.2 General Procedure for Synthesis of Building Blocks

To a solution of corresponding acid (1 equiv) in CH_2Cl_2 was added *N*-[3-(dimethylamino) propyl]-*N*-ethylcarbodiimide (1 equiv), 1-Hydroxybenzotriazole (1 equiv) and the appropriate amine (1 equiv). The mixture was stirred at r.t. and the product formation was monitored by TLC. The reaction

was quenched with water, and the product was extracted with CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The amides were purified by flash-column chromatography.

2.2.3 General Procedure for Preparation of Galantamine Derivatives

Appropriate bromo-amide (1.1 equiv) and anhydrous K_2CO_3 (3 equiv) were added to a solution of norgalanthamine (1 equiv) in anhydrous acetonitrile (2 mL) under argon atmosphere. After stirring at 60 °C for 24 h, the solvent was removed *in vacuo*. The residue was directly subjected to purification by flash column chromatography on silica gel (CH_2Cl_2/MeOH/NH_4OH) to give desired products.

2.3 Assessment of AChE Inhibitory Activity

AChE activity was assayed as described by Ellman *et al.*[72] with some modifications.[73] Fifty μL of *Electrophorus electricus* AChE (Sigma-Aldrich) in buffer phosphate (pH 7.6) and 50 μL of the tested compounds (0.2–300 μM in methanol) were dissolved in 700 μL in the same buffer. The mixtures were incubated for 30 minutes at room temperature before the addition of 100 μL of the substrate solution (0.5 M DTNB, 0.6 mM ATCI in buffer, pH 7.6). The absorbance was read in Shimadzu spectrophotometer at 405 nm after three minutes. Enzyme activity was calculated as a percentage compared to an assay using a buffer without any inhibitor using nonlinear regression. IC_{50} values are means ± SD of three individual determinations each performed in triplicate.

2.4 Assessment of Neurotoxicity on NEURO-2A Cells

Murine neuroblastoma NEURO-2A cells (German collection DSMZ in Braunschweig, Germany) were cultivated under standard conditions: complete medium (90% DMEM, 10% heat inactivated FBS and 1x non-essential aminoacids); 37 °C and 5% CO_2 in fully humidified atmosphere. The cell line was kept in the logarithmic growth phase by splitting 1:4 once a week using trypsin/EDTA. About 30% of the cells grow like neuronal cells. For the experimental evaluation of the cytotoxicicty NEURO-2A cells were plated in 96-well flat bottomed cell culture plates at the recommended density of 1×10^6 cells/25 cm^2. Twenty four hours later cells were treated with various concentrations of the investigational compounds. After 72 h incubation a MTT dye reduction assay was performed.[74] Briefly, at the end of incubation a MTT stock solution (10 mg/ml in PBS) was added (10 μl/well). Plates were further incubated at 37 °C for 4 h. Next, the formazan crystals were dissolved by the addition of 110 μl/well 5% formic acid in 2-propanol (v/v). Absorption was measured at 580 nm wave length on an automated ELISA reader Labexim LMR1. At least 6 wells per concentration were used and data were processed using the GraphPad Prism 5.0 software.2 Heading 1st Order.

3 Results and Discussion

3.1 Design of GAL Derivatives with Dual-site Binding

Five series (**1**–**5**) with alkylamide spacers and different aromatic fragments were designed (Figure 1). Each series consisted of four members with different length of the alkylamide spacers. Members **a** contained a butylamide linker, members **b** – a pentylamide, members **c** – a hexylamide, and members **d** – a heptylamide. The aromatic fragment in the compounds of series **1** was phenyl, in series **2** – benzyl, in series **3** – phenethyl, in series **4** – phenylglycine methyl ester, and in series **5** – phenylalanine methyl ester.

3.2 Molecular Docking of the Designed Derivatives and AChE Inhibitory Activity Prediction

The 20 newly designed compounds were docked into the *rh*AChE (pdb code: 4EY6)[66] by GOLD v. 5.1[67] using a previously optimized docking protocol[52,68] as described in Methods. The only difference in the present protocol was the scoring function used. Here, we used the novel ChemPLP function in GOLD which is 5 times faster than GoldScore and recent validation tests have shown it to be generally more effective than the other scoring functions for both

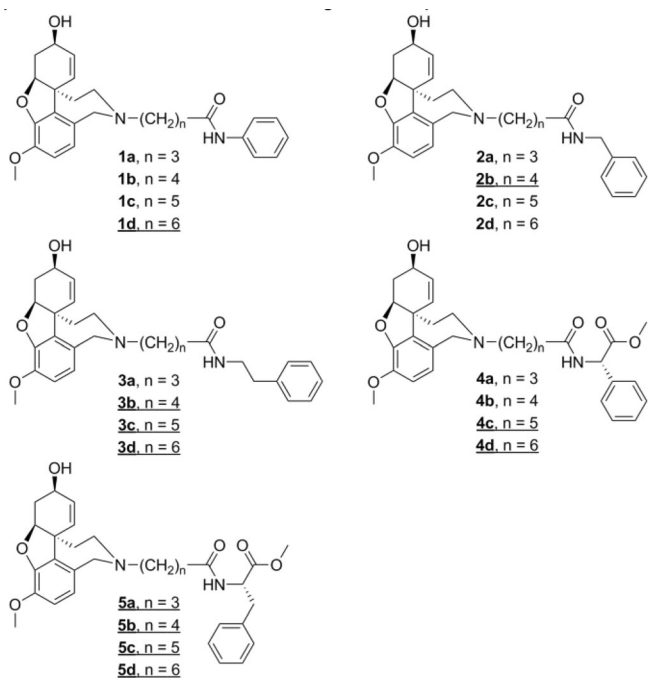


Figure 1. GAL derivatives with dual-site binding, designed in the present study. The underlined structures were synthesized and tested for AChE inhibitory activity.

pose prediction and virtual screening.[56] The pK_a values of the

novel compounds range from 7.52 to 7.93, i.e. at physiological pH part of the molecules are protonated. The X-ray structures of the complexes between *Torpedo californica* AChE and three GAL derivatives show that both axial and equatorial orientations are possible to bind inside the gorge.[49,50] We modeled four sets of compounds for docking: equatorial protonated, equatorial non-protonated, axial protonated and axial non-protonated. The average scores of the highest scoring poses from 3 runs with 10 poses each of all docked compounds from each set were as follows: 103.24, 105.92, 101.72 and 103.02, respectively. The scores were very close and intercorrelated (data not shown). No robust conclusion about the preferred orientation of the alkylamide spacers could be drawn. The scores of the equatorial protonated set are given in Table 1.

The ChemPLP scores reveal a clear relationship: score increases when the length of alkylamide spacer increases. This relationship was expected because longer chains mean more interactions along the AChE gorge. The molecular weights vary from 434.53 (**1a**) to 562.70 (**5d**) (Table 1). The logP values are between 2.80 (**2a**) and 4.24 (**3d**). All compounds contain 2 hydrogen bond donors. The compounds with and without methyl carboxylate fragment have 8 and 6 hydrogen-bond acceptors, respectively. The Lipinski's rule is fulfilled for the novel compounds with the only exception of molecular mass which is slightly higher than 500 for some of the compounds. The BBB permeability was predicted by two algorithms (SVM and LiCABEDS[70]) applied on four types of fingerprints and eight different predictions were generated. Series **1a–d**, **2a–d** and **3a–d** were predicted as BBB permeable (BBB+) by all eight methods, while series **4a–d** and **5a–d** were predicted as BBB non-permeable (BBB−) by some of the methods (Table 1). The presence of methyl ester group might affect negatively the BBB permeability of compounds.

As series **3** and **5** were among the highest scored compounds without and with methyl carboxylate fragment, respectively, they were selected in full for synthesis. One or two compounds were selected randomly from the other three series.

3.3 Synthesis of the Best Predicted GAL Derivatives

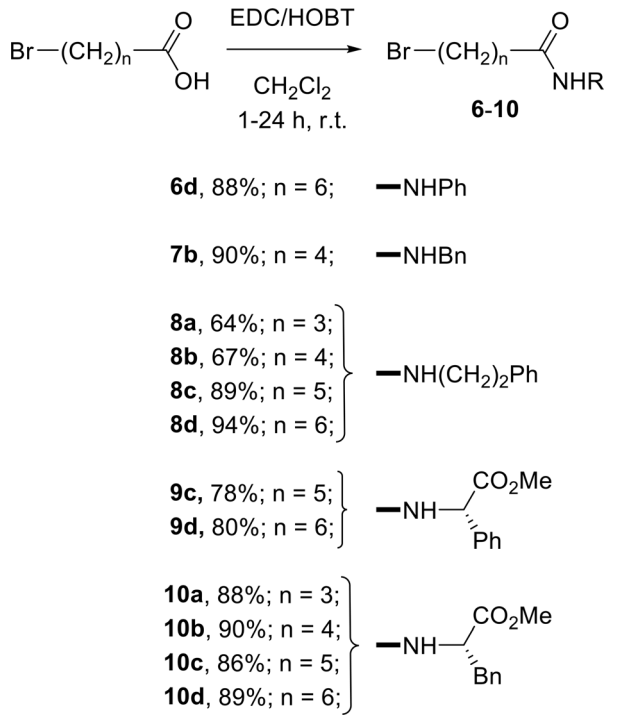
The target molecules were synthesised in two steps: preparation of bromo-amides and their subsequent reaction with *N*-demethylated galantamine.

The first step was performed by reacting the commercially available 4-bromobutanoic acid, 5-bromopentanoic acid, 6-bromohexanoic acid, or 7-bromoheptanoic acid with aniline, benzylamine, phenethylamine, phenylglycine methyl

Table 1. ChemPLP scores, experimental IC_{50} and pIC_{50} values, physicochemical properties, predicted BBB permeability and neurotoxicity of the newly designed GAL derivatives. The underlined compounds were synthesized and tested for AChE inhibitory activity.

Comp.	ChemPLP score	IC_{50} μM (exp) EeAChE*	Times more active than GAL	pIC_{50} (exp)	Mw	logP	H-bond donors	H-bond acceptors	BBB permeability****	Neuro toxicity IC_{50} μM
1a	94.45				434.53	3.14	2	6	8	
1b	97.67				448.55	3.44	2	6	8	
1c	98.38				462.58	3.67	2	6	8	
1d	100.87	0.0169 ± 0.0054	63	7.772	476.61	4.20	2	6	8	> 50
2a	93.69				448.55	2.80	2	6	8	
2b	99.82	0.0308 ± 0.0025	35	7.512	462.58	3.10	2	6	8	> 50
2c	101.61				476.61	3.33	2	6	8	
2d	104.84				490.63	3.86	2	6	8	
3a	100.55				462.58	3.18	2	6	8	
3b	101.79	0.0308 ± 0.0026	35	7.512	476.61	3.48	2	6	8	> 50
3c	106.09	0.0210 ± 0.0016	51	7.679	490.63	3.71	2	6	8	> 50
3d	109.94	0.0008 ± 0.00003	1338	9.097	504.66	4.24	2	6	8	> 50
4a	103.16				506.59	2.99	2	8	6	
4b	105.50				520.62	3.29	2	8	5	
4c	107.52	0.0527 ± 0.0025	20	7.278	534.64	3.52	2	8	7	> 50
4d	111.86	0.0211 ± 0.0027	51	7.677	548.67	4.05	2	8	7	> 50
5a	97.53	0.0958 ± 0.0017	11	7.019	520.62	3.14	2	8	7	> 50
5b	104.38	0.0264 ± 0.0023	40	7.578	534.64	3.44	2	8	6	> 50
5c	108.24	0.0246 ± 0.0016	43	7.609	548.67	3.67	2	8	7	> 50
5d	116.95	0.0011 ± 0.0007	1008	8.974	562.70	4.20	2	8	7	> 50
GAL HBr	74.56	1.070 ± 0.0738	1	5.971	287.35**	1.75	1	4	8	> 50
*r****				0.828						

* Recalculated at GAL IC_{50} = 1.07 μM. ** Molecular mass of GAL base. *** Correlation coefficient between ChemPLP scores and pIC_{50} values. **** Predictions made by BBB Predictor (http://www.cbligand.org/BBB/). The integer shows the number of models giving positive prediction (BBB+).

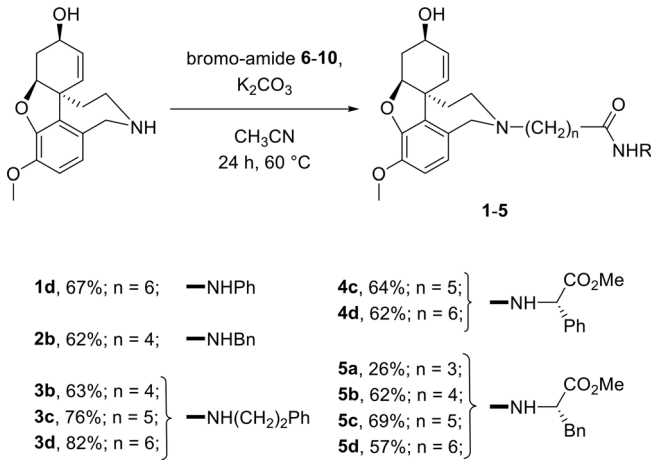


Scheme 1. Synthesis of the bromo-amide intermediates.

ester, or phenylalanine methyl ester. The chosen combinations were performed, using the reagents for peptide synthesis 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 1-Hydroxybenzotriazole hydrate (HOBT), resulting into the desired bromo-amides **6**–**10** (Scheme 1).

Amides **8a** and **10a** were unstable under the reaction conditions and storage at room temperature. Hence, they were prepared by shortening the reaction times, and after quick purification on silica gel were stored at −10 °C.

The galantamine was demethylated following a known procedure.[75] The reaction of norgalantamine with the initially prepared bromides **6**–**10** in dry acetonitrile, and potassium carbonate as a base led to the set of target compounds **1**–**5** (Scheme 2).



Scheme 2. Synthesis of the target compounds.

Unfortunately, all attempts to prepare the galantamine derivative **3a** were unsuccessful. The problem appeared due to the unstable starting bromo-amide **8a** under the reaction conditions. The low yield of **5a** was explained also with the low stability of its precursor **10a** when heated under basic conditions. All other substances were isolated in moderate to good yields after flash column chromatography and were fully characterized. Details about the synthesized compounds are given in Supporting Information 1. The ^{1}H and ^{13}C NMR spectra of the synthesized compounds are given in Supporting Information 2.

3.4 Assessment of AChE Inhibitory Activity and Neurotoxicity

The inhibitory potential of the novel GAL derivatives against *Electrophorus electricus* (electric eel) AChE was tested according to the methodology developed by Ellman *et al.*[72] with some modifications.[73] The UniProt alignment of rhAChE (P22303) and EeACh (O42275) shows that all 17 amino acids forming the binding gorges are identical. The aligned structures are given as Supporting Information 3. On this basis, we use the predictions made on *rh*AChE to assess experimentally the anti-AChE activity of novel compounds on *Ee*AChE. GAL was used as a positive control and the enzyme activity was calculated at $IC_{50} = 1.07$ μM for GAL. The IC_{50} values (μM) of the novel compounds are shown in Table 1. The relationship between the ChemPLP scores and the length of linkers was confirmed by the experimental IC_{50} values. The IC_{50} values decrease gradually from members **a** to members **d** in both **3** and **5** series. Compounds **3d** and **5d** demonstrated the highest acetylcholinesterase inhibitory activities among the novel derivatives, i.e. 0.8 nM and 1.1 nM, respectively. They are 1338 and 1008 times, respectively, more active than GAL. The rest compounds showed AChE inhibitory activities between 9 and 63 times higher than this of GAL ($IC_{50} = 1.070$ μM). Good correlation ($r = 0.828$) was found between the ChemPLP scores and the pIC_{50} ($-\log IC_{50}$) values of the novel GAL derivatives. All compounds were non-toxic on NEURO-2A cells. The IC_{50} values exceeded 50 μM as estimated by MTT-dye reduction assay.

The highest scored poses of **3d** and **5d** are visualized in Figure 2. They are superposed with AChE – Aβ complex, derived previously by RosettaDock.[68,76] It is seen that both novel GAL derivatives fill well the binding gorge. The absence of methyl carboxylate fragment in **3d** allows the phenyl ring to adopt a conformation sterically hindered for Aβ, while the presence of this group in **5d** prevents from adopting such conformation. Additionally, the terminal phenyl ring in **3d** takes part in a sandwich-type π-π interaction with Tyr72 and His287 (Figure 3). In this sense, the GAL derivatives with flexible heptylamide spacer ending with

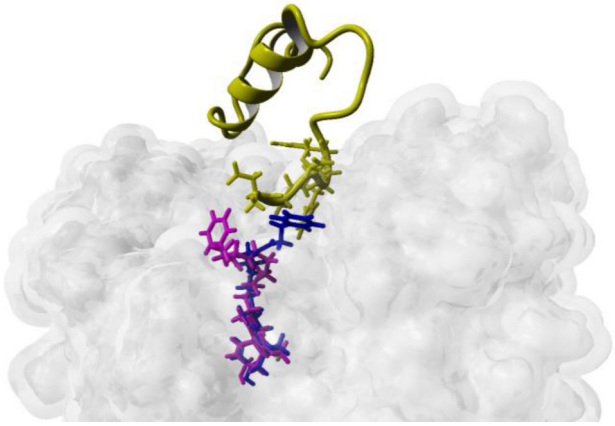


Figure 2. The highest scored poses of 3d (magenta) and 5d (blue) superposed with AChE – Aβ complex. Aβ is given as yellow ribbon. The solvent accessible surface of AChE is given in grey.

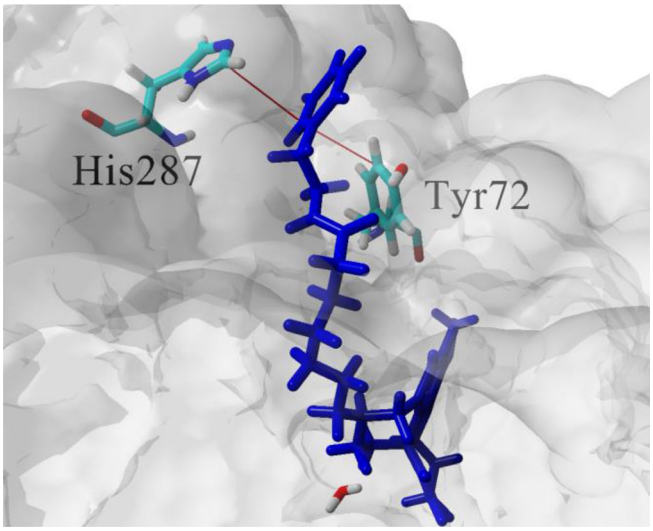


Figure 3. The terminal phenyl ring in **3d** (blue) takes part in a sandwich-type π-π interaction with Tyr72 and His287. The solvent accessible surface of AChE is given in grey.

phenethyl fragment are perspective AChE inhibitors with dual-site binding.

4 Conclusions

The molecular docking-based predictions are a useful tool in the design of novel GAL derivatives with dual-site binding fragments. The synthesized and tested novel compounds confirm the predictions. The heptylamide spacer is long enough to bridge the GAL moiety bound in the catalytic site and the aromatic fragments interacting with PAS. Among the tested terminal aromatic fragments, the phenethyl substituent is the most suitable for binding in PAS. The presence of a methyl carboxylate group in a close proximity to the aromatic fragment affects unfavourably the binding conformation.

Conflict of Interest

None declared.

Supporting Information

Supporting Information 1: Data for the newly synthesized compounds in the present study.

Supporting Information 2: ^{1}H and ^{13}C NMR spectra of the synthesized compounds 1–5.

Supporting Information 3: Multiple sequence alignment of hAChE (UniProt P22303) and EeAChE (UniProt O42275) using CLUSTAL O (1.2.1).

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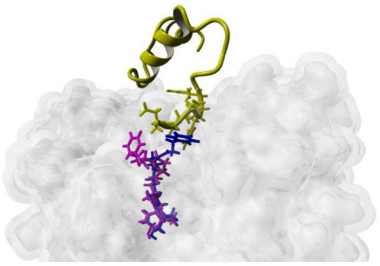

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Docking-based Design of Galantamine Derivatives with Dual-site Binding to Acetylcholinesterase