Chapter 6

Galantamine Derivatives as Acetylcholinesterase Inhibitors: Docking, Design, Synthesis, and Inhibitory Activity

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Abstract

Galantamine (GAL) is a well-known acetylcholinesterase (AChE) inhibitor, and it is widely used for treatment of Alzheimer's disease. GAL fits well in the catalytic site of AChE, but it is too short to block the peripheral anionic site (PAS) of the enzyme, where the amyloid beta (Aβ) peptide binds and initiates the Aβ aggregation. Here, we describe a docking-based technique for designing of GAL derivatives with dual-site binding fragments – one blocking the catalytic site and another blocking the PAS. The highly scored compounds are synthesized and tested. Protocols for docking, design, synthesis, and AChE inhibitory test are given.

Key words Galantamine, Acetylcholinesterase inhibition, Docking, Drug design, Dual-site binding

1 Introduction

The binding site of recombinant human AChE (*rh*AChE) consists of several domains [1–5]. The catalytic anionic site (CAS) lies on the bottom of 20 Å deep and narrow binding gorge. It consists of the catalytic triad: Ser203, Glu334, and His447. The anionic domain binds the quaternary trimethylammonium choline moiety of acetylcholine (ACh). Despite its name, it does not contain any anionic residues but only aromatic ones: Trp86, Tyr130, Tyr337, and Phe338. They are involved in cation-pi interactions with the protonated head of ACh. The acyl pocket consists of two bulky residues, Phe295 and Phe297, and it determines the selective binding of ACh by preventing the access of larger choline esters. The oxyanion hole hosts one molecule of structural water and consists of Gly121, Gly122, and Ala204. The water molecule bridges the binding between enzyme and substrate by hydrogen-bond networking and stabilizes the substrate tetrahedral transition state.

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Finally, the peripheral anionic site (PAS) lies at the entrance to binding gorge. It is composed of five residues: Tyr72, Asp74, Tyr124, Trp286, and Tyr341. PAS allosterically modulates catalysis [6] and is implicated in non-cholinergic functions such as amyloid deposition [7], cell adhesion, and neurite outgrowth [8, 9]. It was found that the Aβ peptide binds close to PAS, interacting hydrophobically with residues 275–305 [7]. This interaction promotes the formation of amyloid fibrils characteristic of Alzheimer's disease [10, 11]. The blockade of PAS prevents the AChE-induced Aβ aggregation [7]. This pivotal finding prompted the design of novel AChEIs with dual binding moieties – one blocking the CAS at the bottom of the gorge and one blocking the PAS [12–17].

Galantamine (GAL) is an alkaloid initially isolated from the bulbs and flowers of *Galanthus caucasicus*, *Galanthus woronowii* (Amaryllidaceae), and related genera [18]. Paskov first developed GAL as an industrial drug under the trade name Nivalin (Sopharma, Bulgaria) [19]. It has been used for treatment of myasthenia gravis, myopathy, residual poliomyelitis paralysis syndromes, sensory and motor disorders of CNS, and decurarization [20–24]. Because of its ability to cross the blood-brain barrier and to affect the central cholinergic function, in the 1980s, GAL was investigated for treatment of Alzheimer's disease (AD) and in 2000 was approved for use in Europe, the United States, and Asia [25–27].

In addition to its acetylcholinesterase (AChE)-inhibiting ability, GAL has been identified as an allosteric modulator of nicotinic acetylcholine receptors (nAChRs) [28–31]. The stimulation of nAChRs can increase intracellular Ca^{2+} levels and facilitate noradrenaline release; both effects enhance the cognitive brain function [32]. Recently, Takata et al. have demonstrated that treatment of rat microglia with GAL significantly enhanced microglial amyloid-β (Aβ) phagocytosis and facilitated Aβ clearance in brains of rodent AD models [33]. This multiple-target action of GAL makes it a most valuable drug for AD treatment and prompts the synthesis of novel GAL derivatives with improved binding to AChE [33–39]. Several series of GAL derivatives with dual-site binding to the enzyme have been prepared and tested [16, 36, 37, 40–42]. All of them showed good AChE inhibitory activities.

In the present paper, we summarize our experience in developing of galantamine derivatives as acetylcholinesterase inhibitors with dual-site binding [43–45]. We used docking-based design in a stepwise manner to develop derivatives with binding affinity more than 1000 times higher than that of GAL.

2 Materials

2.1 Docking Software

GOLD [46] is a docking tool based on a genetic algorithm, and it has proven successful in virtual screening, lead optimization, and identification of the correct binding mode of active molecules [47, 48]. GOLD takes into account the flexibility of the ligand as well as the flexibility of the residues in the binding site. GOLD v. 5.1 was used in our studies.

2.2 Reactants for Synthesis

Fine chemicals: 5-hydroxyindole, 97%; 5-aminoindole, 97%; 4-hydroxybenzaldehyde, 99%; 1,6-dibromohexane, 98%; 7-bromoheptanoic acid, 97%; 6-bromohexanoic acid, 98%; 5-bromovaleric acid, 97%; 4-bromobutyric acid, 98%; aniline, 99.8%; benzylamine, 99%; phenethylamine, 99%; (S)-(+)-2-phenylglycine methyl ester hydrochloride, 97%; L-phenylalanine methyl ester hydrochloride, 98%; L-tryptophan methyl ester hydrochloride, 98%; galantamine hydrobromide, 95%.

Reagents: potassium carbonate, 99 + %; sodium triacetoxyborohydride, 97%; *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), 98%; 1-hydroxybenzotriazole hydrate, 99%; *N*,*N*-diisopropylethylamine, for synthesis; 3-chloroperoxybenzoic acid, 70–75%; ammonia water, 25%, pure for analysis; iron(II) sulfate heptahydrate, 99 + %; sodium sulfate, anhydrous, 99 + %; sodium bicarbonate, 99 + %; triethylamine, 99%; hydrochloric acid, ca. 37% solution in water.

Solvents: 1,2-dichloroethane, extra pure; acetonitrile, 99.9%, extra dry over molecular sieve; methylene chloride, extra pure; petroleum ether 40–60 °C, extra pure; ethyl acetate, for analysis; methanol for HPLC.

2.3 Reactants for AChE Inhibition Assay

Electrophorus electricus AChE (Sigma-Aldrich), acetylthiocholine (ATCl), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), phosphate buffer (pH 7.6), UV-Vis Spectrophotometer Shimadzu UV-1203 at 405 nm.

3 Methods

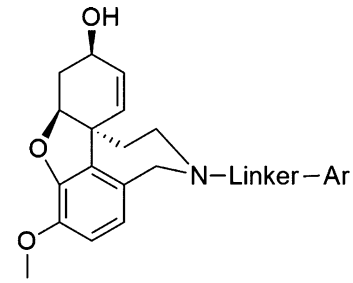
3.1 Design

The GAL derivatives are N-substituted products (Fig. 1). Linkers of different type and length connect the GAL core to aromatic fragments. The GAL core fits into the catalytic site, while the aromatic fragment aims to block the PAS (Fig. 2).

3.2 Molecular Docking Protocol

The optimized docking protocol includes the following settings:

1. Protein: X-ray structure of rhAChE in complex with galantamine (GAL) (pdb id: 4EY6, R = 2.15 Å) [49]. The ligand is removed.



Linkers: $-(CH_2)_nO-$, $-(CH_2)_nCONH-$, $(CH_2)_nCONH(CH_2)_n$,
$-(CH_2)_nCONH(CH_2)_nCH(COOCH_3)-$
n = 3, 4, 5, 6
Ar: indolyl, phenyl

Fig. 1 N-substituted GAL derivatives

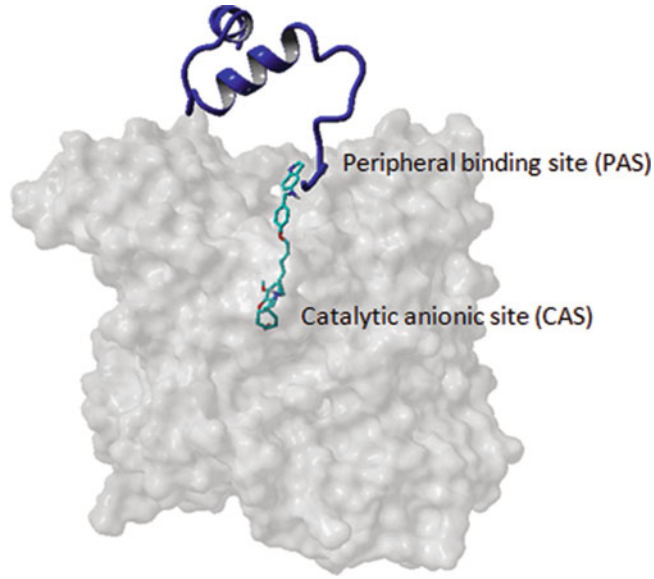


Fig. 2 The GAL core fits into the catalytic site, while the aromatic fragment blocks the PAS. AChE given as *gray* surface, inhibitor in *cyan*, and amyloid beta peptide in *blue*

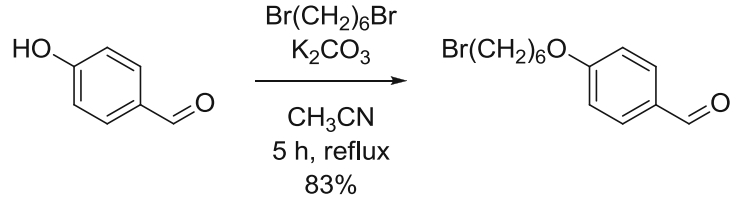
2. Ligands: The structures of the designed molecules are entered in .mol2 format.
3. Scoring function: GoldScore or ChemPLP.
4. Radius of the binding site: 10Å.
5. One water molecule (HOH846) is kept within the binding site.
6. Flexible side chains in the binding site: Tyr72, Asp74, Trp86, Tyr124, Ser125, Trp286, Phe297, Tyr337, Phe338, and Tyr341.

The compounds with the highest GoldScores or ChemPLP scores are selected for synthesis.

3.3 Synthesis

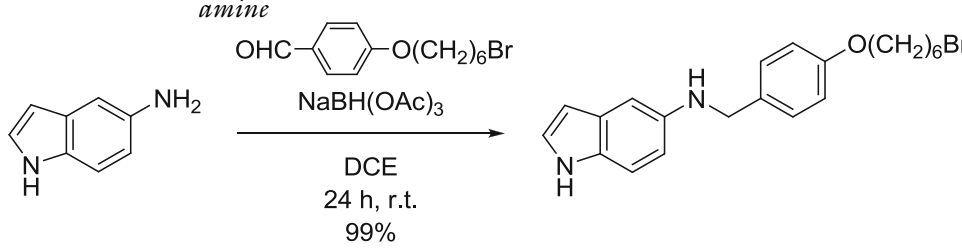
3.3.1 Synthesis of Bromo-Containing Linkers of Different Type and Length Connected to Aromatic Fragments: Br-Linker-Ar

Protocol 1: *Synthesis of 4-(6-bromohexyloxy)benzaldehyde*



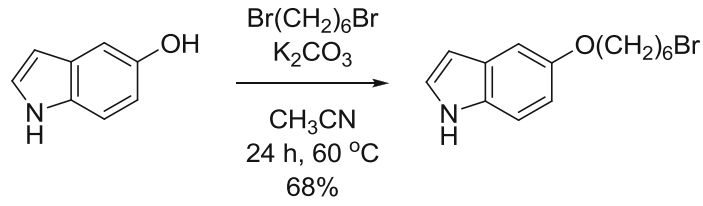
1. *Load a 100 ml round bottom flask with 4-hydroxybenzaldehyde (0.5 g, 4.1 mmol), K_2CO_3 (2.83 g, 20.5 mmol), and CH_3CN (20 ml).*
2. *Add to the stirred suspension 1,6-dibromohexane (12.3 mmol, 1.86 ml), equip the flask with a condenser, and reflux for 5 hours.*
3. *Stop the heating, remove the condenser, and concentrate till dry under reduced pressure.*
4. *Separate the residue between ethyl acetate and water, extract the aqueous phase with ethyl acetate, and dry the organic extracts over Na_2SO_4. Filter from the solids and concentrate.*
5. *Purify* via *flash column chromatography using silica gel as stationary phase and a mixture of ethyl acetate/petroleum ether (1:9) as mobile phase.*

Protocol 2: *Synthesis of* N*-(4-(6-bromohexyloxy)benzyl)-1*H*-indol-5-amine*



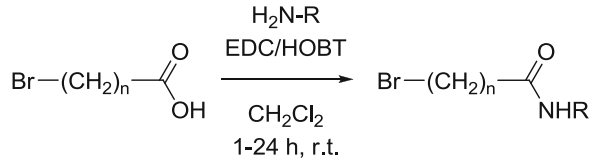
1. *Load a 50 ml round bottom flask with 1*H*-indol-5-amine (0.100 g, 0.757 mmol), 4-(6-bromohexyloxy)benzaldehyde (0.216 g, 0.757 mmol), and 1,2-dichloroethane (8 ml).*
2. *Stir the mixture for 30 min at room temperature, add NaBH $(OAc)_3$ (0.240 g, 1.136 mmol), and continue the stirring for 24 h.*
3. *Quench the reaction with sat.aq.$NaHCO_3$ (50 ml), extract the product with CH_2Cl_2, and dry the organic extracts over Na_2SO_4. Filter from the solids and concentrate.*
4. *Purify* via *flash column chromatography using silica gel as stationary phase and a mixture of petroleum ether/ethyl acetate/triethylamine (2:1:0.5) as mobile phase.*

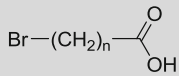
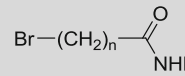
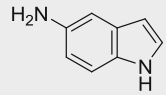
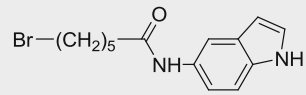
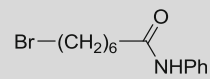
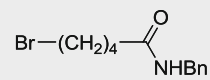
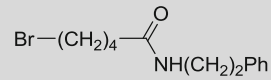
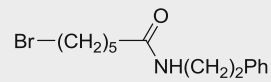
Protocol 3: *Synthesis of 5-(6-bromohexyloxy)-1H-indole*



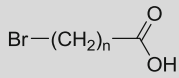
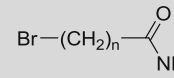
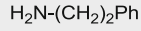
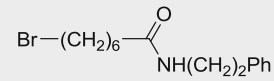
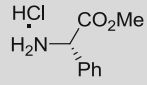
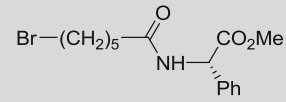
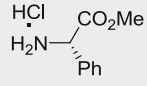
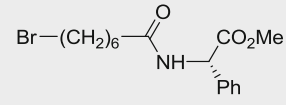
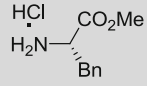
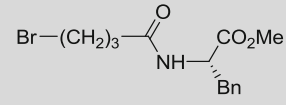
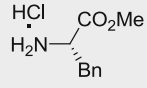
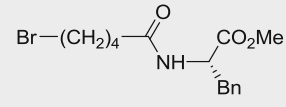
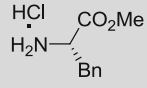
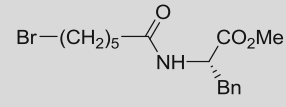
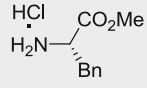
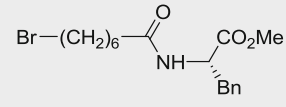
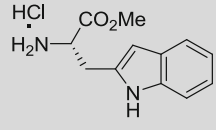
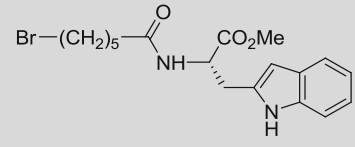
1. *Load a 50 ml round bottom flask with 1H-indol-5-ol (0.100 g, 0.750 mmol), CH_3CN (15 ml), and K_2CO_3 (0.310 g, 2.250 mmol).*
2. *Add to the stirred suspension 1,6-dibromohexane (0.220 g, 0.900 mmol) and heat at 60 °C for 24 h.*
3. *Cool to room temperature, filter through a pad of Celite, and concentrate till dry under reduced pressure.*
4. *Purify* via *flash column chromatography using silica gel as stationary phase and a mixture of petroleum ether/ethyl acetate (4:1) as mobile phase.*

Protocol 4: *Synthesis of bromo-amide intermediates*



Bromo carboxylic acid 	Appropriate amine H_2N-R	Product (bromo-amide intermediate) 	Yield %
$n = 5$			84
$n = 6$	H_2N-Ph		88
$n = 4$	H_2N-Bn		90
$n = 4$	H_2N-$(CH_2)_2$Ph		67
$n = 5$	H_2N-$(CH_2)_2$Ph		89

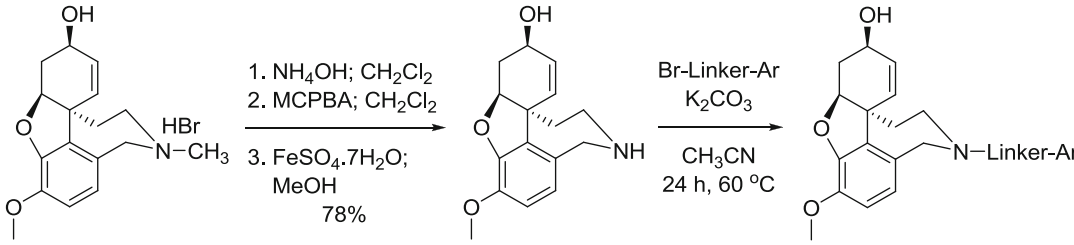
(continued)

Bromo carboxylic acid 	**Appropriate amine** 	**Product (bromo-amide intermediate)** 	**Yield %**
$n = 6$			94
$n = 5$			78
$n = 6$			80
$n = 3$			88
$n = 4$			90
$n = 5$			86
$n = 6$			89
$n = 5$			99

1. *Load a 50 ml round bottom flask with bromo carboxylic acid (1.1 mmol), CH_2Cl_2(20 ml),* N*-[3-(dimethylamino)propyl]-*N*-ethylcarbodiimide (0.210 g, 1.1 mmol), 1-hydroxybenzotriazole (0.148 g, 1.1 mmol), and the appropriate amine (1 mmol).*
2. *In the cases of amino acid methyl ester hydrochlorides, add also* N*,* N*-diisopropylethylamine (1.1 mmol).*
3. *Stir the mixture at room temperature. Follow the reaction development by TLC.*
4. *Concentrate under reduced pressure till dry.*
5. *Purify* via *flash column chromatography using silica gel as stationary phase and a mixture of dichloromethane/ethyl acetate (20:1) as mobile phase.*

3.3.2 Synthesis of the Target Compounds

Protocol 5: *Synthesis of norgalantamine and its subsequent alkylation with Br-Linker-Ar:*



1. *To a stirred suspension of galantamine hydrobromide (0.734 g, 2 mmol) in dichloromethane (10 ml), add 25% aqueous NH_4OH (1 ml) and stir till clear. Separate the organic layer, dry it over Na_2SO_4, and concentrate under reduced pressure to isolate free galantamine base.*
2. *Dissolve the galantamine (2 mmol) into dichloromethane (10 ml), add portionwise 3-chloroperoxybenzoic acid (0.542 g, 2.2 mmol), and stir at room temperature for 1 h.*
3. *Cool the mixture to 0 °C and add methanol (20 ml) followed by $FeSO_4.7H_2O$ (0.556 g, 2 mmol). Stir for ½ h at 0 °C and for 3 h at room temperature.*
4. *Quench the reaction with 5 N HCl (40 ml), remove the organic solvents under reduced pressure, and wash the aqueous residue twice with ether.*
5. *Cool the aqueous layer to 0 °C and basify it with 25% aqueous NH_4OH.*
6. *Extract with dichloromethane, dry over Na_2SO_4, and concentrate.*

7. *Purify* via *flash column chromatography using silica gel as stationary phase and a mixture of dichloromethane/methanol (5:1) as mobile phase to isolate norgalantamine.*
8. *Prepare under argon atmosphere a solution of norgalantamine (0.200 g, 0.732 mmol) in anhydrous acetonitrile (25 mL).*
9. *Add the appropriate Br-Linker-Arom (0.951 mmol) and anhydrous K_2CO_3 (0.303 g, 2.2 mmol).*
10. *Stir at 60 °C for 24 h, cool to room temperature, filter through a pad of Celite, and remove the solvent under reduced pressure.*
11. *Purify* via *flash column chromatography using silica gel as stationary phase and a mixture of dichloromethane/methanol/ammonia (20:1:0.05) to isolate the desired product.*

Structure elucidation: *The structures of the newly synthesized compounds were confirmed by 1D and 2D NMR spectra. Their purity was proven by elemental analysis or HRMS. The spectral analyses were in accordance with the assigned structures. Additionally, the compounds were characterized by melting points and specific rotation for the chiral compounds.*

3.4 AChE Inhibition Assay

The AChE activity is assayed as described by Ellman et al. [50] with some modifications [51]. 50 μL of *E. electricus* AChE (Sigma-Aldrich) in buffer phosphate (pH 7.6) and 50 μL of the test compounds (4–500 μM in methanol) dissolved in 700 μL in the same buffer are mixed. The mixtures are incubated for 30 min at room temperature. 100 μL of the substrate solution (0.5 M DTNB, 0.6 mM ATCI in buffer, pH 7.6) is added. The absorbance is read in Shimadzu spectrophotometer at 405 nm after 3 min. Enzyme activity is 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. GAL is used as a positive control.

4 Some Results Illustrating the Docking-Based Design Efficiency

In order to illustrate the efficiency of the proposed docking-based design of novel dual-site binding GAL derivatives, in Table 1., we present some results from our in-house database of GAL derivatives designed to block simultaneously the CAS and PAS [44, 45]. More than hundred compounds were designed and docked into AChE and the best scored of them were synthesized and tested.

Most of the compounds were 10–100 times more active than GAL. However, two of them – compounds *9* and *15* – were more than 1000 times more active. They are subject to further research.

Table 1
Docking scores and experimental IC_{50} values of the designed GAL derivatives

Comp.	Linker	Ar	Docking score[a]	IC_{50} (exp) μM	Times more active than GAL
1	$(CH_2)_6O$	Phenyl-CH_2NH-indolyl	119.15	0.011	95
2	$(CH_2)_6O$	Indolyl	112.32	0.012	93
3	$(CH_2)_5CONH$	Indolyl	109.06	0.015	72
4	$(CH_2)_5CONHCH(COOCH_3)CH_2$	Indolyl	113.89	0.094	11
5	$(CH_2)_6CONH$	Phenyl	100.87	0.0169	63
6	$(CH_2)_4CONHCH_2$	Phenyl	99.82	0.0308	35
7	$(CH_2)_4CONH(CH_2)_2$	Phenyl	101.79	0.0308	35
8	$(CH_2)_5CONH(CH_2)_2$	Phenyl	106.09	0.021	51
9	$(CH_2)_6CONH(CH_2)_2$	Phenyl	109.94	0.0008	1338
10	$(CH_2)_5CONHCH(COOCH_3)$	Phenyl	107.52	0.0527	20
11	$(CH_2)_6CONHCH(COOCH_3)$	Phenyl	111.86	0.0211	51
12	$(CH_2)_3CONHCH(COOCH_3)CH_2$	Phenyl	97.53	0.0958	11
13	$(CH_2)_4CONHCH(COOCH_3)CH_2$	Phenyl	104.38	0.0264	40
14	$(CH_2)_5CONHCH(COOCH_3)CH_2$	Phenyl	108.24	0.0246	43
15	$(CH_2)_6CONHCH(COOCH_3)CH_2$	Phenyl	116.95	0.0011	1008
GAL HBr			74.56	1.07	1

Data are taken from Refs. [44, 45]
[a]GoldScores for compounds *1–4*
GAL HBr ChemPLP scores for compounds *5–15*

5 Conclusions

Molecular docking is a widely used structure-based method for virtual screening of huge databases of compounds as well as for binding prediction of newly designed ligands. It was used solely or in combination with 2D- and 3D-QSAR or machine learning methods [52–64]. Here, we described a protocol for molecular docking of GAL derivatives into the enzyme AChE. The protocol was optimized stepwise to find the settings (scoring function,

flexible binding site, radius of the binding site, presence/absence of a water molecule) correlating best with the binding affinities of compounds. The optimized protocol was used to predict the affinities of newly designed GAL derivatives with dual-site binding to AChE. The best scored compounds were synthesized and tested. All of them are 10–100 times more active than GAL. Even more, two of them are more than 1000 times more active.

6 Note

The BLAST alignment of AChEs from human, rat, rabbit, and *E. electricus* shows that the main residues forming the binding site are conserved.

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