

Novel Cholinesterase Inhibitors Based on *O*-Aromatic *N,N*-Disubstituted Carbamates and Thiocarbamates

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Abstract: Based on the presence of carbamoyl moiety, twenty salicylanilide *N,N*-disubstituted (thio)carbamates were investigated using Ellman's method for their ability to inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). *O*-Aromatic (thio)carbamates exhibited weak to moderate inhibition of both cholinesterases with IC₅₀ values within the range of 1.60 to 311.0 μM. IC₅₀ values for BChE were mostly lower than those obtained for AChE; four derivatives showed distinct selectivity for BChE. All of the (thio)carbamates produced a stronger inhibition of AChE than rivastigmine, and five of them inhibited BChE more effectively than both established drugs rivastigmine and galantamine. In general, 5-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide, 2-hydroxy-*N*-phenylbenzamide as well as *N*-methyl-*N*-phenyl carbamate derivatives led to the more potent inhibition. *O*-{4-Chloro-2-[(4-chlorophenyl)carbamoyl]phenyl} dimethylcarbamothioate was identified as the most effective AChE inhibitor (IC₅₀ = 38.98 μM), while 2-(phenylcarbamoyl)phenyl diphenylcarbamate produced the lowest IC₅₀ value for BChE (1.60 μM). Results from molecular docking studies suggest that carbamate compounds, especially *N,N*-diphenyl substituted representatives with considerable portion of aromatic moieties may work as non-covalent inhibitors displaying many interactions at peripheral anionic sites of both enzymes. Mild cytotoxicity for HepG2 cells and consequent satisfactory calculated selectivity indexes qualify several derivatives for further optimization.

Keywords: acetylcholinesterase; butyrylcholinesterase; carbamate; enzyme inhibition; salicylanilide; thiocarbamate

1. Introduction

Acetylcholine (ACh) is a cholinergic neurotransmitter interacting with either nicotinic or muscarinic receptors, e.g., at all autonomic ganglia, neuromuscular junction, the preganglionic sympathetic and parasympathetic neurons, in all the parasympathetic innervated organs, adrenal medulla, and at the sweat glands. The effect of ACh in synapses is terminated by the action of acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (pseudocholinesterase or plasma cholinesterase; BChE; EC 3.1.1.8), which hydrolyse ACh rapidly into choline and acetate. However, AChE remains the major cholinesterase within the human brain. This enzyme exists in multiple

molecular forms with similar catalytic properties. The esteratic site, where ACh is hydrolysed, contains the catalytic triad of three amino acids: serine, histidine and glutamate [1–3].

Degeneration of the cholinergic projection from the *nucleus basalis Meynerti* to the forebrain neocortex and associated limbic structures is one of the pathologies associated with Alzheimer's disease (AD) [3], the most common type of dementia. Unfortunately, the number of patients suffering from this disease has increased continuously during the last decades. Based on cholinergic hypothesis, inhibition of cholinesterases (ChE) represents the mainstay of pharmacotherapy of AD. During the progression of AD, BChE activity and BChE/AChE ratio are increased thus compensating the loss of AChE activity. BChE can substitute for AChE constitutively. That is why BChE inhibition may provide a desirable feature of AD therapy [1,4]. Current drugs for the treatment of AD are non-selective (rivastigmine) or AChE-selective inhibitors (galantamine, donepezil) [1,5]. However, these three ChE inhibitors possess clinically comparable efficacy and side-effect profiles. It is unclear whether additional inhibition of BCHE by rivastigmine or galantamine's allosteric modulation of nicotinic receptors offer any extra clinical benefit [6]. In addition to increased ACh levels and symptomatic benefit, recent findings indicate that ChE inhibitors can attenuate neuronal damage and protect them from cellular death. This way might affect AD pathogenesis and slow down the progression [7], but there is still no really effective causative therapy.

It has been demonstrated that carbamate moiety is a pharmacophore for ChE inhibition activity. Various carbamates have been proposed and proven as highly efficient AChE and BChE inhibitors [8–12]. Also rivastigmine ((S)-3-[1-(dimethylamino)ethyl]phenyl ethyl(methyl)carbamate; Figure 1 used in the treatment of AD and some other dementias belong to aromatic carbamates. In addition to this use, carbamates have been used in human medicine (e.g., for the treatment of myasthenia gravis, glaucoma), veterinary medicine, agriculture, and for military purposes [3].

The mechanism of action of carbamates is distinct from the majority of organophosphorus-based inhibitors, although both these groups are substrate analogues of ACh. They enter the active site and affect the esteratic site of ChE by covalent interaction (phosphorylation or carbamoylation) with serine hydroxyl providing more stable esters than those resulted from physiological reaction (acetylation) with ACh. The principal difference between organophosphates and carbamates consists in the stability of the ChE-inhibitor complex. Organophosphates phosphorylate serine residues in a non-reversible way and the regeneration of the enzyme is very slow (irreversible inhibition). Less stable carbamoyl-serine complex can be split by spontaneous hydrolysis markedly faster (pseudo-irreversible inhibition) [3,13]. Depending on the structure of carbamates, ChE inhibition can be also irreversible or non-covalent [10,14,15].

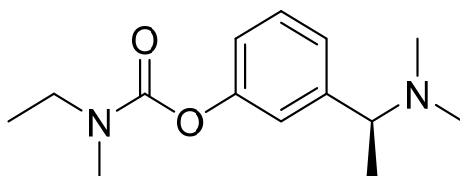


Figure 1. Rivastigmine.

Salicylanilide derivatives exhibit a wide range of interesting biological properties including inhibition of various enzymes [16–22]. Previously, we identified salicylanilide *O,O*-diethyl thiophosphates (phosphorothioates) [23] and their oxo analogues (diethyl phosphates) [24] as pseudo-irreversible inhibitors of both AChE and BChE with low micromolar IC_{50} values. Interestingly, thiophosphates exhibited the balanced activity against both cholinesterases [23] whereas phosphates showed more effective inhibition of BChE [24]. Imramovský *et al.* [14] reported inhibition of AChE by salicylanilide *N*-alkyl carbamates and their molecular docking.

Keeping in mind these facts about carbamates and salicylanilides, we evaluated salicylanilide *N,N*-disubstituted (thio)carbamates as potential inhibitors of both ChE. Previously, these (2-(phenylcarbamoyl)phenyl disubstituted(thio)carbamate molecules (their general structure is depicted in

Figure 2) exhibit *in vitro* antimicrobial activity against mycobacteria, Gram-positive cocci and *Trichophyton mentagrophytes*. In contrast to parent salicylanilides, these compounds offer a lower cytotoxicity and a higher lipophilicity expressed as calculated $\log P$ values [19]. Increased $\log P$ may help to improve poor bioavailability of salicylanilides and to facilitate permeability through the biological membranes [16]. More lipophilic derivatives are also able to enter the central nervous system via passive diffusion more easily and/or readily.

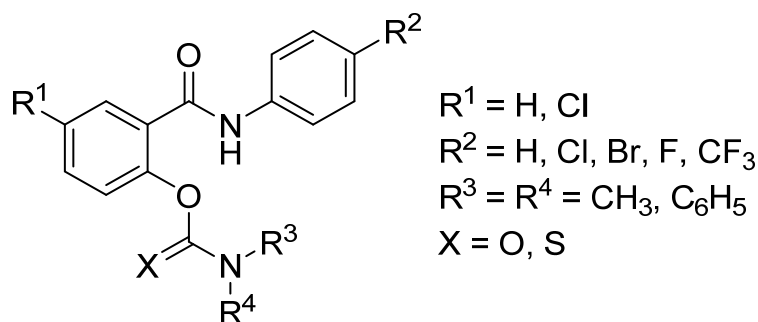


Figure 2. Salicylanilide (thio)carbamates **1a–5d**.

2. Results and Discussion

2.1. *In Vitro* Inhibition of Acetylcholinesterase and Butyrylcholinesterase

The IC_{50} values were determined using the spectrophotometric Ellman's method, which is a simple and rapid method to determine the SH and -S-S- groups content in proteins [25]. This method is widely used for the evaluation of cholinesterase activity and screening the efficiency of ChE inhibitors. Cholinesterase activity is measured indirectly by quantifying the concentration of the 5-mercapto-2-nitrobenzoic acid ion formed in the reaction between the thiol reagent 5,5'-dithiobis(2-nitrobenzoic acid) and thiocholine, a product of substrate hydrolysis (*i.e.*, acetylthiocholine, ATCh) by cholinesterases [26]. Ellman's method was modified slightly according to Zdrzilova *et al.* [27].

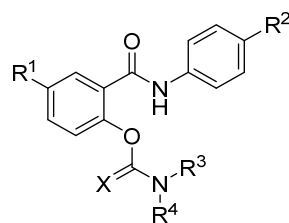
The inhibitory potency of fifteen carbamates and five thiocarbamates was evaluated for AChE from electric eel and BChE from equine serum. The efficacy of the screened compounds is expressed as IC_{50} values, representing the concentration of an inhibitor required for 50% inhibition of the enzyme. The results were compared with rivastigmine and galantamine, two established anti-dementia drugs with different structures and mechanisms of action. Rivastigmine is an acylating pseudo-irreversible carbamate-based inhibitor affecting the function of both cholinesterases, while galantamine acts as a non-acylating competitive reversible inhibitor. Furthermore, it modulates allosterically nicotinic ACh receptors.

The synthesis of (thio)carbamates **1a–5d** was reported previously by Krátký *et al.* [19].

All of the (thio)carbamates **1a–5d** showed weak to moderate inhibition of both cholinesterases with IC_{50} values from 1.60 to 311.0 μM (Table 1). The IC_{50} values of all tested derivatives for inhibition of AChE were in a comparatively narrow concentration range of 38–90 μM . *O*-{4-Chloro-2-[(4-chlorophenyl)carbamoyl]phenyl} dimethylcarbamothioate (**1b**) was identified as the most effective AChE inhibitor ($\text{IC}_{50} = 38.98 \mu\text{M}$), whereas *O*-{4-chloro-2-[(4-fluorophenyl)carbamoyl]phenyl} dimethylcarbamothioate (**3b**) produced the least inhibition with IC_{50} of 89.74 μM . In general, it is possible to conclude that better effectiveness was achieved by the 4-(trifluoromethyl)aniline and 5-chlorosalicylic acid derivatives **4a–d**, although compared to other derivatives the IC_{50} value differences are not enormous. That is why there is no clear influence of carbamate nitrogen substitution pattern on the activity. The removal of 5-chlorine atom from salicylic moiety is well tolerated without any increase of IC_{50} values (derivatives **5** *vs.* **1–4**) similarly to the replacement of carbamoyl by thiocarbamoyl group and *vice versa* (compounds **b** *vs.* **a, c, d**). Nevertheless, regarding a large number of agents with their activity in low micromolar, submicromolar and nanomolar

concentrations [2,8,12], investigated derivatives **1a–5d** could be considered to be only weak AChE inhibitors.

Table 1. IC₅₀ values for acetylcholinesterase and butyrylcholinesterase.



Code	R ¹	R ²	R ³	R ⁴	X	IC ₅₀ for AChE [μM]	IC ₅₀ for BChE [μM]	Selectivity to BChE
1a	Cl	Cl	CH ₃	CH ₃	O	72.87 ± 1.06	88.42 ± 1.27	0.8
1b	Cl	Cl	CH ₃	CH ₃	S	38.98 ± 1.11	51.38 ± 1.65	0.8
1c	Cl	Cl	CH ₃	Ph	O	74.83 ± 2.95	4.20 ± 0.18	17.8
1d	Cl	Cl	Ph	Ph	O	54.81 ± 1.43	106.46 ± 3.64	0.5
2a	Cl	Br	CH ₃	CH ₃	O	62.38 ± 1.36	54.21 ± 0.30	1.2
2b	Cl	Br	CH ₃	CH ₃	S	59.05 ± 2.13	54.64 ± 0.42	1.1
2c	Cl	Br	CH ₃	Ph	O	81.60 ± 2.92	4.30 ± 0.51	19.0
2d	Cl	Br	Ph	Ph	O	63.29 ± 0.95	144.80 ± 29.02	0.4
3a	Cl	F	CH ₃	CH ₃	O	76.17 ± 2.87	311.00 ± 4.50	0.2
3b	Cl	F	CH ₃	CH ₃	S	89.74 ± 4.68	91.37 ± 5.45	1.0
3c	Cl	F	CH ₃	Ph	O	62.54 ± 5.03	7.34 ± 0.14	8.5
3d	Cl	F	Ph	Ph	O	49.03 ± 3.88	15.63 ± 0.11	3.1
4a	Cl	CF ₃	CH ₃	CH ₃	O	49.04 ± 0.54	28.14 ± 0.86	1.7
4b	Cl	CF ₃	CH ₃	CH ₃	S	41.98 ± 0.55	36.22 ± 0.28	1.2
4c	Cl	CF ₃	CH ₃	Ph	O	49.85 ± 2.76	1.97 ± 0.02	25.3
4d	Cl	CF ₃	Ph	Ph	O	47.39 ± 2.78	49.00 ± 0.17	1.0
5a	H	H	CH ₃	CH ₃	O	57.74 ± 1.60	83.59 ± 6.84	0.7
5b	H	H	CH ₃	CH ₃	S	61.94 ± 6.56	26.62 ± 2.78	2.3
5c	H	H	CH ₃	Ph	O	55.01 ± 2.46	11.38 ± 0.48	4.8
5d	H	H	Ph	Ph	O	53.79 ± 4.26	1.60 ± 0.01	33.6
	Rivastigmine					501 ± 3.08	19.95 ± 0.20	25.1
	Galantamine					4 ± 0.13	7.96 ± 0.59	0.5

AChE and BChE inhibition is expressed as the mean ± SD ($n = 3$ experiments). Selectivity to BChE: IC₅₀ for AChE/IC₅₀ for BChE. The best results for each enzyme and favourable selectivity are shown in bold.

In contrast to AChE, IC₅₀ values for BChE are in a broader range of 1.60 to 311.0 μM. Among all of the tested compounds, 2-(phenylcarbamoyl)phenyl diphenylcarbamate (**5d**) was found to be the most potent BChE inhibitor *in vitro* (IC₅₀ value of 1.60 μM) and the least effective inhibitor was 4-chloro-2-[(4-fluorophenyl)carbamoyl]phenyl dimethylcarbamate (**3a**, IC₅₀ = 311.0 μM). The most active BChE inhibitors displayed moderate inhibitory action, remaining activities are rather weak. Similarly to AChE inhibition, improved activity towards BChE was shown by derivatives of 4-(trifluoromethyl)aniline **4a–d**; their IC₅₀ values did not exceed the value of 50 μM. Moreover, this group includes the second best inhibitor of BChE from all of the tested molecules, methyl(phenyl)carbamate **4c** with IC₅₀ of 1.97 μM. Aniline ring substitution by 4-chlorine or 4-bromine produced a similar activity, which was superior to 4-fluoroaniline derivatives. Interestingly, non-halogenated (thio)carbamates **5** exhibited also a significant inhibition suggesting that halogenation of both salicylic and aniline rings is not necessary for the activity. Focusing on the various (thio)carbamate types used for the modification of halogenated salicylanilides, the compounds were ordered based on decreasing inhibition activity against BChE as follows: *N*-methyl-*N*-phenyl carbamates **c** >> *N,N*-dimethyl thiocarbamates **b** > *N,N*-

diphenyl carbamates **d** and *N,N*-dimethyl carbamates **a**. For the majority of derivatives, the change of *N,N*-dimethyl carbamates **a** to thiocarbamates **b** as well as the replacement of one *N*-methyl by phenyl increased the activity.

When concentrated on selectivity to ChE (Table 1), (thio)carbamates can be classified into three groups. Members of first group are more effective inhibitors of AChE (selectivity to BChE of ≤ 0.5 ; **1d**, **2d**, **3a**). Second, the largest group embraces compounds with balanced inhibition of both enzymes (selectivity within the range of 0.5 to 2.0): **1a**, **1b**, **2a**, **2b**, **3b**, **4a**, **4b**, **4d**, **5a**, in general *N,N*-dimethyl (thio)carbamates. Remaining derivatives including all *N*-methyl-*N*-phenyl carbamates are stronger inhibitors of BChE: **1c**, **2c**, **3c**, **3d**, **4c**, **5b-d**. Selectivity of four of them (**1c**, **2c**, **4c** and especially **5d**) towards BChE exceeded the value of 10, thus being comparable with rivastigmine.

The activities of salicylanilide derivatives **1a–5d** were compared with those of galantamine and rivastigmine. All of the tested derivatives showed a greater inhibition of AChE than rivastigmine but not galantamine, and five of them (**1c**, **2c**, **3c**, **4c** and **5d**) inhibited BChE more effectively than both drugs; additionally, two carbamates (**3d**, **5c**) exhibited superiority to rivastigmine only.

Drawing a comparison between *N,N*-disubstituted (thio)carbamates **1a–5d** and previously reported salicylanilide-based cholinesterases inhibitors, *N*-alkyl carbamates [14] demonstrated equal or almost equal IC_{50} values for AChE. Salicylanilide diethyl phosphates [24] showed a similar activity against AChE, but they inhibited BChE slightly stronger and more selectively. Diethyl thiophosphates (phosphorothioates) were superior towards AChE and they exhibited a comparatively closer concentration range for the inhibition of BChE. However, the most active carbamates **1c**, **2c**, **3c**, **4c**, **5d** showed lower IC_{50} values than the most active thiophosphates.

2.2. Cytotoxicity and Selectivity Indexes of (Thio)Carbamates

We also investigated selectivity indexes (SI) for (thio)carbamates inhibitors. SI is calculated as a ratio of cellular toxicity and IC_{50} values (in μM) for enzymes, and SI value higher than 10 indicates a rather acceptable toxicity (based on the analogy of the therapeutic index). Cytotoxicity was determined in human hepatocellular carcinoma (HepG2) cell model previously [19]. For the calculation of selectivity indexes, we selected several carbamates and thiocarbamates. First, the most effective inhibitors of AChE and BChE were involved (**1b**, **4b** and **1c**, **2c**, **3c**, **4c**, **5d**, respectively) and then two carbamates (**4a**, **5a**) with the lowest toxicity were added (Table 2).

In general, all SI values for thiocarbamates **1b** and **4b** are poor due to their comparatively higher cytotoxicity (83.51 and 67.29 μM , respectively). Selectivity indexes of **1c**, **3c**, **4b**, **4c** and **5d** for this enzyme did not reach breakpoint of 10 due to the lower activity against AChE. On the other hand, two dimethylcarbamates with IC_{50} values ~ 6 mM (**4a**, **5a**) provided a satisfactory selectivity (≥ 103.7), followed by *N*-methyl-*N*-phenyl carbamate **2c** (SI of 11.6). With respect to BChE, seven derivatives from total nine showed sufficient SI values within the range of >50.8 with superiority of 2-[(4-bromophenyl)carbamoyl]-4-chlorophenyl methyl(phenyl)carbamate (**2c**) and 4-chloro-2-[[4-(trifluoromethyl)phenyl]carbamoyl]phenyl dimethylcarbamate (**4a**, SI ≥ 219.8), thus indicated a safety of investigated derivatives.

Table 2. Cytotoxicity and selectivity indexes for selected (thio)carbamates.

Code	IC_{50} for HepG2 [μM] [19]	Selectivity Index for AChE	Selectivity Index for BChE
1b	83.51	2.1	1.6
1c	289.0	3.9	68.8
2c	945.1	11.6	219.8
3c	512.0	8.2	69.8
4a	6508.0	132.7	231.3
4b	67.29	1.6	1.9
4c	>100	>2.0	>50.8
5a	5985.9	103.7	71.6
5d	211.1	3.9	131.9

Selectivity indexes higher than 10 are shown in bold.

2.3. Molecular Docking

In order to elucidate possible mode of binding, molecular docking was performed using crystallographic structures of human AChE (pdb code 4PQE) and human BChE (pdb code 1POI). In the preparation process all water molecules were removed from the enzymes and structures of enzymes and ligands were optimized using UCSF Chimera software package (Amber force field) [28]. Docking was performed using Autodock Vina [29] and Autodock 4.2 [30]. The molecules of enzymes were partly flexible. The 3D affinity grid box was designed to include the full active and peripheral site of AChE and BChE.

Generally, carbamate compounds are considered to be covalent inhibitors reacting directly with the active-site serine hydroxyl [3,13]. However, being rather spacious molecules and with their carbamate bond relatively inaccessible for the direct interaction with the enzyme (especially for diphenyl substituted carbamates **d**), compounds **1a–5d** prepared in our laboratory could also act as non-covalent or allosteric inhibitors. Moreover, the presence of four aromatic moieties could be beneficial for the formation of interactions in the active gorge of AChE which is lined predominantly by aromatic amino acid residues or interactions with the peripheral anionic site (PAS) of both AChE and BChE. Additionally, some carbamates have been also reported as the PAS-directed irreversible inhibitors of AChE [15] or proposed as competitive acting agents [14].

The structures of AChE and BChE active sites have been studied extensively and are described in detail [31–33]. Active sites are buried under the surface of the molecules at the bottom of a relatively narrow gorge [31]. Though both enzymes share considerable structure similarities, there are significant differences resulting in distinct susceptibility to substrates and modulators of activity.

The active gorge of AChE is lined with aromatic acid residues serving for the navigation of positively charged molecules towards the esteratic site. In close proximity of catalytic triad Ser-His-Glu, several aromatic acid residues (e.g., Trp86, Phe337) can be found, forming so-called α -anionic site [32]. Moreover, on the outer rim of the cavity, another region consisting of predominantly aromatic residues (Trp286, Tyr72, Tyr124) comprises peripheral anionic site. This structure feature is important for the function of allosteric inhibitors [34]. The active site of BChE has very similar organisation, though its cavity is composed mostly of hydrophobic residues of aliphatic amino acids which leaves more space in the gorge increasing thus the ability to accommodate larger molecules with bulkier substituents [35,36].

Molecular modelling studies performed on AChE with the series of salicylanilide *N,N*-disubstituted (thio)carbamates **1a–5d** suggested that these compounds fill the cavity of the enzyme with aniline part of the molecule heading out of the gorge, salicylic aromatic ring in close proximity to Trp86 of α -anionic site and amide group pointing towards Ser203 hydroxyl. All of the molecules displayed a similar orientation though diphenyl substituted carbamates (**1a–5d**) stayed closer to the enzyme surface showing additional interactions with PAS.

Two modes of binding were observed in the active site of BChE. *N,N*-Dimethyl carbamates **a**, thiocarbamates **b** and one representative of *N*-methyl-*N*-phenyl carbamate (**5c**) showed a similar orientation spreading across the cavity, with salicylic part of the molecule showing π - π interactions with Trp82 and Tyr332 and aniline ring displaying π - π interactions with Phe329 (T-stacking) and amide bond in relatively close proximity to Ser198 (O-N = 3.20 Å for the compound **5c**). *N,N*-Diphenyl carbamates **d** and some of *N*-methyl-*N*-phenyl carbamates **c** appeared to be moved slightly out of the cavity blocking substantially the entrance to the gorge. Diphenylcarbamoyl part of the molecule fills up a small aromatic hollow formed by Trp82, Tyr332, Trp430 and Tyr440, carbonyl oxygene is in close proximity to Ser198 (O-O = 2.83 Å), salicylic moiety shows a possible interaction with Phe329 and aniline part of the molecule is heading out of the gorge (Figure 3).

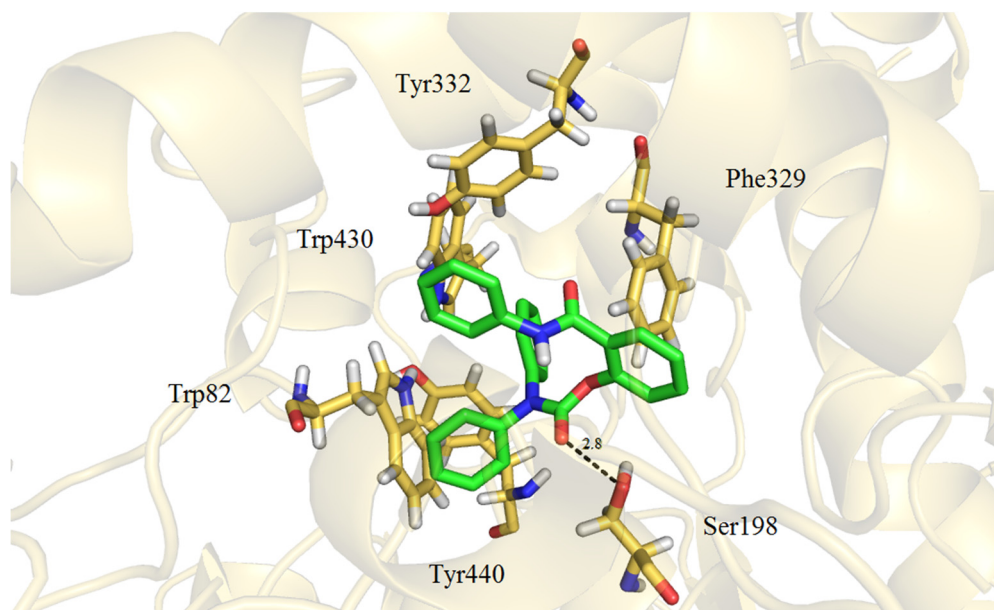


Figure 3. Compound **5d** in active site of BChE together with interacting residues.

3. Experimental Section

3.1. Determination of Cholinesterase Inhibition

The IC_{50} values were determined using the spectrophotometric Ellman's method [25,26]. All of the tested compounds were dissolved in 0.01 M dimethyl sulphoxide and then diluted in demineralised water to 0.001 M and 0.0001 M. Ellman's method was modified slightly according to Zdrzilova *et al.* [27]. Acetylcholinesterase was obtained from electric eel (*Electrophorus electricus* L.) and butyrylcholinesterase was from equine serum. Galantamine and rivastigmine were involved as reference drugs.

3.2. Molecular Docking

Molecular docking was performed using crystallographic structures of human AChE (pdb code 4PQE) and human BChE (pdb code 1POI). The 3D structures of ligands were prepared in Chem3D Pro 13.0 (a part of ChemBioOffice 2012 Package, CambridgeSoft, Cambridge, MA, USA). In the preparation process all water molecules were removed from the enzymes and structures of enzymes and ligands were optimized using UCSF Chimera software package (Amber force field) [28]. Docking was performed using Autodock Vina [29] and Autodock 4.2 [30] (a Lamarckian genetic algorithm was used). The molecules of enzymes were partly flexible. The 3D affinity grid box was designed to include the full active and peripheral site of AChE and BChE. The number of grid points in the x -, y - and z -axes was 24, 24 and 24 with grid points separated by 1 Å (Autodock Vina) and 40, 40 and 40 with grid points separated by 0.4 Å (Autodock 4).

4. Conclusions

Fifteen salicylanilide N,N -disubstituted carbamates and five N,N -dimethylthiocarbamates were evaluated for their inhibition of acetylcholinesterase from electric eel and butyrylcholinesterase from equine serum. All derivatives exhibited *in vitro* inhibition of both ChE with micromolar and low micromolar IC_{50} values. Several derivatives showed a promising activity especially against BChE. Eight derivatives were more selective against BChE, whereas remaining compounds produced mostly balanced inhibition of both enzymes. (Thio)carbamates share higher *in vitro* activity when compared to rivastigmine against AChE (IC_{50} values of ≤ 89.7 and 501 μ M, respectively) and seven of them even against BChE (*i.e.*, with IC_{50} lower than 19.95 μ M). N -Methyl- N -phenyl carbamates and derivatives of 5-chloro-2-hydroxy- N -[4-(trifluoromethyl)phenyl]benzamide were identified optimal for the enzyme inhibition. Selected salicylanilide N,N -disubstituted (thio)carbamates also exhibited

satisfactory selectivity indexes for both cholinesterases as a consequence of their comparatively mild cytotoxicity.

Molecular docking studies performed on both enzymes suggest that these molecules may act as non-covalent inhibitors of AChE and BChE. Especially *N,N*-diphenyl substituted carbamates showed many possible interactions in the PAS blocking thus the entrance of the enzyme cavity. The preference of *N*-methyl-*N*-phenyl and *N,N*-diphenyl carbamates towards BChE at the expense of AChE springs from the differences in the active gorge arrangement. Though cavity of AChE contains a significant amount of aromatic acid residues, it is possible that BChE with more spacious cavity may be able to accommodate compounds with larger substituents optimally.

These results can stimulate a follow-up study as its starting point with the goal to reach more efficacious inhibitors with submicromolar activities keeping also the low cytotoxicity.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ACh: acetylcholine

ATCh: acetylthiocholine

AD: Alzheimer's disease

AChE: acetylcholinesterase

BChE: butyrylcholinesterase

ChE: cholinesterases

References

1. Schwarz, S.; Lucas, S.D.; Sommerwerk, S.; Csuk, R. Amino derivatives of glycyrrhetic acid as potential inhibitors of cholinesterases. *Bioorg. Med. Chem.* **2014**, *22*, 3370–3378.
2. Saeed, A.; Mahesar, P.A.; Zaib, S.; Khan, M.S.; Matin, A.; Shahid, M.; Iqbal, J. Synthesis, cytotoxicity and molecular modelling studies of new phenylcinnamide derivatives as potent inhibitors of cholinesterases. *Eur. J. Med. Chem.* **2014**, *78*, 43–53.
3. Colovic, M.B.; Krstic, D.Z.; Lazarevic-Pasti, T.D.; Bondzic, A.M.; Vasic, V.M. Acetylcholinesterase Inhibitors: Pharmacology and Toxicology. *Curr. Neuropharmacol.* **2013**, *11*, 315–335.
4. Mesulam, M.M.; Guillozet, A.; Shaw, P.; Levey, A.; Duysen, E.G.; Lockridge, O. Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. *Neuroscience* **2002**, *110*, 627–639.
5. Ibach, B.; Haen, E. Acetylcholinesterase inhibition in Alzheimer's disease. *Curr. Pharm. Des.* **2004**, *10*, 231–251.
6. Leger, G.C.; Massoud, F. Novel disease-modifying therapeutics for the treatment of Alzheimer's disease. *Expert Rev. Clin. Pharmacol.* **2013**, *6*, 423–442.
7. Francis, P.T.; Nordberg, A.; Arnold, S.E. A preclinical view of cholinesterase inhibitors in neuroprotection: Do they provide more than symptomatic benefits in Alzheimer's disease? *Trends Pharmacol. Sci.* **2005**, *26*, 104–111.
8. Chaudhaery, S.S.; Roy, K.K.; Shakya, N.; Saxena, G.; Sammi, S.R.; Nazir, A.; Nath, C.; Saxena, A.K. Novel Carbamates as Orally Active Acetylcholinesterase Inhibitors Found to Improve Scopolamine-Induced Cognition Impairment: Pharmacophore-Based Virtual Screening, Synthesis, and Pharmacology. *J. Med. Chem.* **2010**, *53*, 6490–6505.

9. Wieckowska, A.; Bajda, M.; Guzior, N.; Malawska, B. Novel alkyl- and arylcarbamate derivatives with *N*-benzylpiperidine and *N*-benzylpiperazine moieties as cholinesterases inhibitors. *Eur. J. Med. Chem.* **2010**, *45*, 5602–5611.
10. Imramovský, A.; Pejchal, V.; Štěpánková, Š.; Vorčáková, K.; Jampílek, J.; Vančo, J.; Šimůnek, P.; Královec, K.; Brůčková, L.; Mandíková, J.; *et al.* Synthesis and *in vitro* evaluation of new derivatives of 2-substituted-6-fluorobenzo[d]thiazoles as cholinesterase inhibitors. *Bioorg. Med. Chem.* **2013**, *21*, 1735–1748.
11. Yuan, W.; Shang, Z.; Qiang, X.; Tan, Z.; Deng, Y. Synthesis of pterostilbene and resveratrol carbamate derivatives as potential dual cholinesterase inhibitors and neuroprotective agents. *Res. Chem. Intermed.* **2014**, *40*, 787–800.
12. Gocer, H.; Akincioglu, A.; Goksu, S.; Gulcin, I.; Supuran, C.T. Carbonic anhydrase and acetylcholinesterase inhibitory effects of carbamates and sulfamoylcarbamates. *J. Enzyme Inhib. Med. Chem.* **2015**, *3*, 316–320.
13. Pohanka, M. Cholinesterases, a target of pharmacology and toxicology. *Biomed. Pap.* **2011**, *155*, 219–229.
14. Imramovsky, A.; Stepankova, S.; Vanco, J.; Pauk, K.; Monreal-Ferriz, J.; Vinsova, J.; Jampilek, J. Acetylcholinesterase-Inhibiting Activity of Salicylanilide *N*-Alkylcarbamates and Their Molecular Docking. *Molecules* **2012**, *17*, 10142–10158.
15. Lin, G.; Lai, C.Y.; Liao, W.C. Molecular Recognition by Acetylcholinesterase at the Peripheral Anionic Site: Structure-Activity Relationships for Inhibitions by Aryl Carbamates. *Bioorg. Med. Chem.* **1999**, *7*, 2683–2689.
16. Krátký, M.; Vinšová, J. Salicylanilide Ester Prodrugs as Potential Antimicrobial Agents—A Review. *Curr. Pharm. Des.* **2011**, *17*, 3494–3505.
17. Krátký, M.; Vinšová, J.; Novotná, E.; Wsól, V.; Ulmann, V.; Stolaříková, J.; Fernandes, S.; Bhat, S.; Liu, J.O. Salicylanilide derivatives block *Mycobacterium tuberculosis* through inhibition of isocitrate lyase and methionine aminopeptidase. *Tuberculosis (Edinb.)* **2012**, *92*, 434–439.
18. Krátký, M.; Vinšová, J.; Novotná, E.; Mandíková, J.; Trejtnar, F.; Stolaříková, J. Antibacterial Activity of Salicylanilide 4-(Trifluoromethyl)benzoates. *Molecules* **2013**, *18*, 3674–3688.
19. Krátký, M.; Volková, M.; Novotná, E.; Trejtnar, F.; Stolaříková, J.; Vinšová, J. Synthesis and Biological Activity of New Salicylanilide *N,N*-Disubstituted Carbamates and Thiocarbamates. *Bioorg. Med. Chem.* **2014**, *22*, 4073–4082.
20. Krátký, M.; Vinšová, J.; Novotná, E.; Stolaříková, J. Salicylanilide pyrazinoates inhibit *in vitro* multidrug-resistant *Mycobacterium tuberculosis* strains, atypical mycobacteria and isocitrate lyase. *Eur. J. Pharm. Sci.* **2014**, *53*, 1–9.
21. Krátký, M.; Novotná, E.; Saxena, S.; Yogeewari, P.; Sriram, D.; Švarcová, M.; Vinšová, J. Salicylanilide Diethyl Phosphates as Potential Inhibitors of Some Mycobacterial Enzymes. *Sci. World J.* **2014**, *2014*, doi:10.1155/2014/703053.
22. Chen, C.L.; Liu, F.L.; Lee, C.C.; Chen, T.C.; Ali, A.A.A.; Sytwu, H.K.; Chang, D.M.; Huang, H.S. Modified Salicylanilide and 3-Phenyl-2*H*-benzo[e][1,3]oxazine-2,4(3*H*)-dione Derivatives as Novel Inhibitors of Osteoclast Differentiation and Bone Resorption. *J. Med. Chem.* **2014**, *57*, 8072–8085.
23. Vinšová, J.; Krátký, M.; Komlóová, M.; Dadapeer, E.; Štěpánková, Š.; Vorčáková, K.; Stolaříková, J. Diethyl 2-(Phenylcarbamoyl)phenyl Phosphorothioates: Synthesis, Antimycobacterial Activity and Cholinesterase Inhibition. *Molecules* **2014**, *19*, 7152–7168.
24. Vinšová, J.; Kozic, J.; Krátký, M.; Stolaříková, J.; Mandíková, J.; Trejtnar, F.; Buchta, V. Salicylanilide diethyl phosphates: Synthesis, antimicrobial activity and cytotoxicity. *Bioorg. Med. Chem.* **2014**, *22*, 728–737.
25. Ou, S.Y.; Kwok, K.C.; Wang, Y.; Bao, H.Y. An improved method to determine SH and -S-S- group content in soymilk protein. *Food Chem.* **2004**, *88*, 317–320.
26. Sinko, G.; Calic, M.; Bosak, A.; Kovarik, Z. Limitation of the Ellman method: Cholinesterase activity measurement in the presence of oximes. *Anal. Biochem.* **2007**, *370*, 223–227.
27. Zdrzilova, P.; Stepankova, S.; Komers, K.; Ventura, K.; Cegan, A. Half-inhibition concentrations of new cholinesterase inhibitors. *Z. Naturforsch. C* **2004**, *59*, 293–296.
28. Pettersen, E.F.; Goddard, T.D.; Huang, C.C.; Couch, G.S.; Greenblatt, D.M.; Meng, E.C.; Ferrin, T.E. UCSF Chimera—A visualization system for exploratory research and analysis. *J. Comput. Chem.* **2004**, *25*, 1605–1612.
29. Trott, O.; Olson, A.J. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **2010**, *31*, 455–461.
30. Morris, G.M.; Huey, R.; Lindstrom, W.; Sanner, M.F.; Belew, R.K.; Goodsell, D.S.; Olson, A.J. Autodock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J. Comput. Chem.* **2009**, *30*, 2785–2791.

31. Sussman, J.L.; Harel, M.; Frolow, F.; Oefner, C.; Goldman, A.; Toker, L.; Silman, I. Atomic structure of acetylcholinesterase from *Torpedo californica*: A prototypic acetylcholine-binding protein. *Science* **1991**, *253*, 872–879.
32. Harel, M.; Schalk, I.; Ehret-Sabatier, L.; Bouet, F.; Goeldner, M.; Hirth, C.; Axelsen, P.; Silman, I.; Sussman, J.L. Quaternary ligand binding to aromatic residues in the active-site gorge of acetylcholinesterase. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 9031–9035.
33. Axelsen, P.H.; Harel, M.; Silman, I.; Sussman, J.L. Structure and dynamics of the active site gorge of acetylcholinesterase: Synergistic use of molecular dynamics simulation and X-ray crystallography. *Protein Sci.* **1994**, *3*, 188–197.
34. Johnson, G.; Moore, S.W. The peripheral anionic site of acetylcholinesterase: Structure, functions and potential role in rational drug design. *Curr. Pharm. Des.* **2006**, *12*, 217–225.
35. Nicolet, Y.; Lockridge, O.; Masson, P.; Fontecilla-Camps, J.C.; Nachon, F. Crystal structure of human butyrylcholinesterase and of its complexes with substrate and products. *J. Biol. Chem.* **2003**, *278*, 41141–41147.
36. Saxena, A.; Redman, A.M.; Jiang, X.; Lockridge, O.; Doctor, B.P. Differences in active-site gorge dimensions of cholinesterases revealed by binding of inhibitors to human butyrylcholinesterase. *Chem. Biol. Interact.* **1999**, *119*, 61–69.

Sample Availability: Samples of the compounds **1a–5d** are available from the authors.



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