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Design, synthesis and evaluation of carbamate-modified (−)-N^1-phenethylnorphysostigmine derivatives as selective butyrylcholinesterase inhibitors

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ABSTRACT

We synthesized carbamate-modified (−)-N^1-phenethylnorphysostigmine derivatives **3a**–**u** and evaluated their anti-cholinesterase activities. In vitro evaluation showed that cyclohexylmethylcarbamate derivative **3u** potently and selectively inhibits butyrylcholinesterase.

Alzheimer's disease (AD) is a progressive and neurodegenerative disease that causes cognitive and memory impairment. It is characterized by senile plaques mainly composed of amyloid beta and neurofibrillary tangles composed of tau protein. Deficiency in cholinergic neurotransmission is, however, the direct cause of cognitive disorders, as evidenced by clinical and experimental reports.[1–4] Intensifying cholinergic transmission is considered to be useful for improving memory functions. Various therapeutic approaches based on the cholinergic hypothesis have been attempted, but only drugs that inhibit the breakdown of acetylcholine (ACh) in the synaptic cleft are clinically approved to date.

ACh released from nerve terminals is rapidly degraded by cholinesterases (ChEs). Because acetylcholinesterase (AChE) is the main player regarding degradation of ACh in normal brain, most work aimed at developing ChE inhibitors has targeted AChE. In AD brain, however, cholinergic neurons are lost with progression of the disease, and AChE is also reduced.[5]

Vertebrates have another acetylcholine hydrolase, butyrylcholinesterase (BuChE). Although it is abundant in plasma, it also exists in the brain. In contrast to AChE, BuChE is mainly derived from glia in the brain.[6] BuChE is therefore believed to not be reduced, and its contribution to degrading ACh tends to be elevated in AD brain.

It has been reported that selective BuChE inhibitors increase brain ACh and augment learning in rodents,[7] and both BuChE knockout mice and silent mutants in humans show no physiological disadvantage,[8,9] inspiring the hypothesis that BuChE may be a promising target in developing anti-AD drugs without serious adverse effects.

There are three important subsites in ChE; anionic site, oxyanion hole, and acyl pocket. Anionic site is mainly composed of Trp86(82) in human AChE (human BuChE), it is important to attract and stabilize the quaternary nitrogen in degradation of ACh. Oxyanion hole is composed of Gly121(116), Gly122(117) and Ala204(199), and it stabilizes carbonyl oxygen of substrates by hydrogen bonding and facilitates the nucleophilic attack on the carbonyl carbon by catalytic Ser203(198). Acyl pocket interacts with acyl moiety of ACh, and among three subsites, its spatial capacity is the most prominent difference between the structures of AChE and BuChE. In AChE, two aromatic amino acids, Phe295 and Phe297, locate in this pocket, but in BuChE, they are replaced by smaller ones, Leu286 and Val288, and this enables BuChE to catalyze various substrates including bulkier esters.

Physostigmine (**1**) is the most classical ChE inhibitor, and its derivatives have been reported to direct their carbamate region to-

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wards the acyl pocket.[10] Regarding the relationship between the carbamate moiety of physostigmine derivatives and anti-ChE activity, the following findings have been reported to date: (i) conversion from methyl group to phenyl or 2-methylphenyl group renders the compounds AChE-selective; (ii) a 4-isopropyl group improves anti-BuChE activity; (iii) substituents on the carbamate moiety greatly affect the duration of the anti-ChE effect.[11] In addition, (−)-N^1-phenethylnorcymserine (PEC, **3d**), which possesses a 4-isopropylphenyl carbamate and N^1-phenethyl group, has been reported to have highly selective and potent anti-BuChE activity.[7]

Because physostigmine (**1**) and its derivatives can exploit the acyl pocket, physostigmine derivatives are considered to be highly useful for developing selective BuChE inhibitors. Although some studies on the structure–activity relationship (SAR) of the carbamate moiety of physostigmine analogs have already been carried out, the variety of substituents was limited to relatively simple ones and some of them were aimed at anti-AChE activity.[10,12–19] An investigation using more diverse derivatives may increase our understanding and aid in the development of more effective compounds. In this report, therefore, we investigated the SAR of the carbamate moiety by synthesizing carbamate-modified derivatives **3a**–**u** of (−)-N^1-phenethylnorphysostigmine. The N^1 moiety was fixed in the shape of a phenethyl group because the N^1-phenethyl group increases the selectivity for BuChE.[20]

As shown in Scheme 1, carbamate-modified (−)-N^1-phenethylnorphysostigmine derivatives **3a–u** were synthesized from N^1-phenethylnoreseroline (**2**), which was prepared from physostigmine (**1**).[21,22] The intermediate **2** was reacted with different R–NCO compounds (**a–u**, Scheme 1) in the presence of catalytic Na[15,16,20,21,23] to generate **3a–u**, including the reported products (−)-N^1-phenethylnorphysostigmine (**3a**), (−)-N^1-phenethylnorphenserine (**3c**) and PEC (**3d**).[21]

Anti-ChE activities were measured using modified Ellman's colorimetric method.[24] Briefly, we used the extracts from mice brain and mice serum as sources of AChE and BuChE, respectively, and all compounds were preincubated with the enzymes for 1 h at 37 °C before the addition of acetylthiocholine or butyrylthiocholine in combination with the coloring reagent 5,5′-dithiobis-(2-nitrobenzoic acid).

The IC_{50} values for AChE and BuChE are summarized in Table 1. In all synthesized derivatives, we fixed the N^1 moiety in the shape of a phenethyl group, which is reported to increase the selectivity for BuChE.[20] In fact, the selectivity was BuChE in all derivatives. The carbamate moiety of physostigmine derivatives is reported to bind in the acyl pocket, and it seems to have an important role in the acquisition of the selectivity for BuChE. Since there is a prominent difference between AChE and BuChE in terms of their spatial volume, we first examined the effect of steric bulk. As in previous reports, the methyl group increased both activities,[17–19] but the *n*-hexyl group selectively reduced the anti-AChE activity by one-fifth (**3a** and **3b**). Regarding substitution on the phenyl ring, the anti-BuChE activity tended to correlate inversely with the steric bulk (**3c**–**i**).

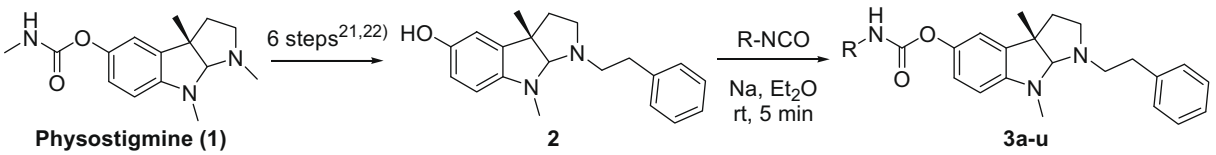
Physostigmine (**1**) and its derivatives inhibit ChEs by carbamoylating the γ-O of the active center Ser. Since the hydroxyl group of the Ser attacks the carbamate in this reaction, the electronic properties of the carbamate moiety may affect the anti-ChE activity. Thus, we also investigated whether the electronic properties influence the activity. The methoxy group at the 4-position of the phenyl ring greatly improved the activity, whereas the *n*-hexanoxy and phenoxy group diminished it, even though they were all electron-donating (**3j**–**l**). Considering that the anti-ChE activity correlated inversely with the steric bulk, these results indicate that

Table 1
Anti-ChE activity and enzyme selectivity

Compounds	R	AChE[a] (nM)	BuChE[a] (nM)	Selectivity[b]
1	Methyl (N^1-methyl, physostigmine)	40	280	0.14
3a	Methyl	170	10	17
3b	*n*-Hexyl	970	14	69
3c	Phenyl	1600	340	5
3d	4-Isopropylphenyl	>100,000	540	>185
3e	4-*t*-Butylphenyl	15,000	430	35
3f	4-*n*-Butylphenyl	48,000	980	49
3g	4-Phenylphenyl	>100,000	1100	>91
3h	4-Dimethylaminophenyl	>100,000	79	>1266
3i	2-Naphthyl	44,000	580	76
3j	4-Methoxyphenyl	33,000	83	398
3k	4-Hexanoxyphenyl	>100,000	1400	>71
3l	4-Phenoxyphenyl	>100,000	2000	>50
3m	3,4-Dimethoxyphenyl	11,000	97	113
3n	5-Benzo[*d*][1,3]dioxolyl	1900	100	19
3o	4-Chlorophenyl	>100,000	370	>270
3p	4-Acetylphenyl	>100,000	5900	>17
3q	4-Nitrophenyl	>100,000	6700	>15
3r	Benzyl	180	6.6	27
3s	Phenethyl	450	16	28
3t	Cyclohexyl	5600	22	255
3u	Cyclohexylmethyl	2500	1.8	1388

[a] To estimate the enzyme activity, changes in the absorbance at 2 min intervals were measured at 415 nm using a spectrophotometer. The enzyme activity is expressed as a percent of the activity of the solvent, DMSO. Seven different concentrations of each compound were used and the IC_{50} values of each compound were calculated by nonlinear regression of the sigmoidal dose–response curve using GraphPad Prism version 4.03. Only the results with correlation coefficients of $r^2 \geqslant 0.95$ were accepted. The results are represented as the mean of the IC_{50} obtained from at least four independent measurements.

[b] The selectivity was calculated as follows; selectivity = IC_{50} AChE/IC_{50} BuChE.



R-NCO :

methyl isocyanate (**a**), *n*-hexyl isocyanate (**b**), phenyl isocyanate (**c**),4-isopropylphenyl isocyanate (**d**), 4-*t*-butylphenyl isocyanate (**e**), 4-*n*-butylphenyl isocyanate (**f**), 4-phenylphenyl isocyanate (**g**), 4-dimethylaminophenyl isocyanate (**h**), 2-naphthyl isocyanate (**i**), 4-methoxyphenyl isocyanate (**j**), 4-hexanoxyphenyl isocyanate (**k**), 4-phenoxyphenyl isocyanate (**l**), 3,4-dimethoxyphenyl isocyanate (**m**), 5-benzo[d][1,3]dioxolyl isocyanate (**n**), 4-chlorophenyl isocyanate (**o**), 4-acetylphenyl isocyanate (**p**), 4-nitrophenyl isocyanate (**q**), benzyl isocyanate (**r**), phenethyl isocyanate (**s**), cyclohexyl isocyanate (**t**), cyclohexylmethyl isocyanate (**u**).

Scheme 1.

the steric factor is more influential than the electronic one. Although the anti-BuChE activities were similar to those of the methoxy substituent, the 3,4-dimetoxyphenyl or cyclic 5-benzo[1,3]dioxolyl substituent enhanced the anti-AChE activity and gave lower selectivity (**3m** and **3n**). Whereas chloro substitution at the 4-position of the phenyl ring maintained the activity, it was decreased by acetyl and nitro group substitution, more potent electron-withdrawing substituents (**3o**–**q**). Overall, the electron-donating property seemed to increase the anti-BuChE activity, whereas the electron-withdrawing property diminished it.

In previous reports, most of the carbamoyl substituents of physostigmine were the *n*-alkyl, phenyl and simply substituted phenyl groups.[10,12,13,17,25] Therefore, we investigated the aralkyl and cycloalkyl groups. The benzyl and phenethyl group greatly increased the anti-BuChE activity, but they concurrently increased the anti-AChE activity and reduced the selectivity (**3r** and **3s**). The cyclohexyl group reduced the anti-AChE activity, whereas high anti-BuChE activity was sustained, resulting in a higher selectivity (**3t**). Considering the results with the phenyl, benzyl and phenethyl group, the compounds having an intervening methylene between the carbamate nitrogen and the ring structure tended to have more potent anti-ChE activities than the directly bound ones. When we applied this trend to the cyclohexyl group, cyclohexylmethyl substitution showed the highest anti-BuChE activity while keeping its high selectivity (**3u**).

Because the carbamoylation is the common event in ChE inhibition by carbamates, similar trend was seen in both AChE and BuChE when the electron-withdrawing substituents were induced (**3p** and **q**) Regarding the enzymatic selectivity, however, steric bulk is seemed to be the most influential factor as expected (**3d**,**g**,**h**). In addition, whereas the phenyl group is planar and considered to increase the anti-AChE activity by π–π interaction with Phe295 and Phe297, cyclohexyl group is voluminous and does not possess π electron, hence it may be difficult to accommodate cyclohexyl group to AChE's strait acyl pocket and anti-AChE activity was decreased. Although we have not conducted a detailed analysis about how this compound interacts with the enzyme, the intervening methylene may confer flexibility to the substituent and enable the carbamate moiety to fit into the acyl pocket more easily.

In conclusion, we synthesized and biologically evaluated diverse carbamate-modified derivatives of (−)-N^1-phenethylnorphysostigmine, and determined that the cyclohexylmethyl group greatly increases the anti-BuChE activity and selectivity for BuChE.

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