Pharmacophore-based design and discovery of (−)-meptazinol carbamates as dual modulators of cholinesterase and amyloidogenesis

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ABSTRACT
Multifunctional carbamate-type acetylcholinesterase (AChE) inhibitors with anti-amyloidogenic properties like phenserine are potential therapeutic agents for Alzheimer’s disease (AD). We reported here the design of new carbamates using pharmacophore model strategy to modulate both cholinesterase and amyloidogenesis. A five-feature pharmacophore model was generated based on 25 carbamate-type training set compounds. (−)-Meptazinol carbamates that superimposed well upon the model were designed and synthesized, which exhibited nanomolar AChE inhibitory potency and good anti-amyloidogenic properties in in vitro test. The phenylcarbamate 43 was highly potent (IC50 31.6 nM) and selectively effective for AChE, and showed low acute toxicity. In enzyme kinetics assay, 43 exhibited uncompetitive inhibition and reacted by pseudo-irreversible mechanism. 43 also showed amyloid-β (Aβ) lowering effects (51.9% decrease of Aβ12) superior to phenserine (31% decrease of total Aβ) in SH-SYSY-APP695 cells at 50 μM. The dual actions of 43 on cholinergic and amyloidogenic pathways indicated potential uses as symptomatic and disease-modifying agents.

Introduction
Alzheimer’s disease (AD) is an age-related neurodegenerative disorder that causes the majority of dementia in the elderly. Pathologically, AD is characterized by the progressive loss of basal forebrain cholinergic neurons, and neuropathological changes of abnormally accumulated extracellular amyloid-β peptide (Aβ)2 and intracellular tau protein. However, the underlying mechanisms of AD are still poorly understood, which may be attributed to the complex multipathogenic features, including amyloidogenic processing of amyloid precursor protein (APP), Aβ aggregation, tau hyperphosphorylation, calcium dyshomeostasis, oxidative stress, mitochondrial dysfunction, deterioration of synaptic neurotransmission, and neuronal apoptosis.

Current approved anti-AD drugs are all palliative treatments targeting cholinergic or glutamatergic neurotransmission thereby symptomatically improving memory and cognition in patients. Acetylcholinesterase (AChE) inhibitors (Figure 1) such as tacrine, donepezil, rivastigmine, and galantamine, are major palliative treatments available now. Carbamates are classical pseudo-irreversible AChE inhibitors, which bind to AChE catalytic site covalently via carbamylating conserved serine residue, and therefore delay the reactivation of an unbound enzyme. Physostigmine (1) is the first AChE inhibitor separated from natural products, but unacceptable toxicity limits its clinical use. Rivastigmine (14) is the only carbamate AChE inhibitor approved as anti-AD drug on the market.

Over the last decade, much effort has been devoted to amyloidogenesis (APP generation/metabolism) and Aβ-induced neurotoxicity. Unfortunately, to date, Aβ-directed therapies, such as γ-secretase inhibitors and immunotherapies, were too toxic to succeed in clinical trials. It seems that the one-molecule-one-target paradigm is inadequate to address the unmet disease-modifying goal of anti-AD drugs. In view of the multifactorial nature of AD pathogenesis, a “multi-target-directed ligands” (MTDLs) strategy was applied in recent development of modifying treatments for AD. Single molecule directing toward different biological targets involved in AD etiology showed promising multipotent profiles. Multifunctional AChE inhibitors with anti-amyloidogenic properties have been most widely studied because of their symptom-alleviating ability and disease-modifying potential.

Phenserine (5), a phenylcarbamate of (−)-eseroline (24), developed by Greig et al., is a unique multipotent AChE inhibitor. It reduces the levels of APP and Aβ via a non-cholinergic mechanism by down-regulating the translation of APP mRNA. The phenylcarbamoyl moiety of phenserine seems to be crucial for its anti-amyloidogenic effect. Although phase III clinical trials of phenserine failed due to lack of efficacy, a redevelopment after correcting some methodological deficiencies might bring to new conclusions.

In our earlier research, bis-(−)-nor-meptazinols and their derivatives were characterized as dual binding site AChE inhibitors with anti-Aβ-aggregation and/or metal-complexing properties. The crystal structure of bis-(−)-nor-meptazinol/AChE complex was resolved, which revealed the binding pose of (−)-meptazinol moiety in the AChE catalytic site. To further explore (−)-meptazinol monomer derivatives, we reported here the design of new...
Pharmacophore modeling

Based on three-dimensional (3D) quantitative structure-activity relationship (QSAR) analysis of 25 known carbamate-type AChE inhibitors, a pharmacophore model was built in silico and validated through a test set of 16 structurally diverse compounds. Guided by the pharmacophore model, (−)-meptazinol was selected as the scaffold to build carbamoyl groups on. Herein, (−)-meptazinol carbamates (42 and 43, Figure 1) were designed and synthesized, and their AChE inhibitory activities were predicted according to ligand pharmacophore mapping. To illustrate potential complementary interactions of the model features with enzyme residues, the pharmacophore model was fitted into the active site of AChE.

Inhibitory potencies of 42 and 43 for AChE and butyrylcholinesterase (BChE) were tested in vitro. Enzyme kinetic parameters, Michaelis constant (K_m) and maximum velocity of reaction (V_max), were measured on recombinant human acetylcholinesterase (rHuAChE). The association and dissociation rate constants, namely inhibition constant (k_i), dissociation constant (k_d), and affinity constant (K_a), were determined using AChE immobilized disk. Anti-amyloidogenic experiments were conducted employing high content screening (HCS) in SH-SYSY-APP_{695} cells, and enzyme-linked immunosorbent assay (ELISA) in the cell culture medium. Mechanisms for the actions of 42 and 43 on reducing APP and Aβ42 levels were further discussed.

Materials and methods

Pharmacophore modeling

Pharmacophore model generation and validation were performed using 3D QSAR Pharmacophore Generation module and Ligand Pharmacophore Mapping module, respectively, in Discovery Studio v2.5 (DS, Accelrys, San Diego, CA) software package. Carbamate-type AChE inhibitors with comparable IC_{50}S tested by Ellman’s method and using physostigmine or rivastigmine as positive control were collected from the literature to generate quantitative pharmacophore hypotheses. The IC_{50} values covered a range of three to four orders of magnitude and the activity uncertainty was set at 3 as default. Two-dimensional (2D) structures of the compounds were built using ISIS Draw v2.2 (MDL Information Systems, Inc.) and exported into DS to be converted into 3D format. A maximum of 255 conformers were generated for each compound over a 20 kcal/mol range using the BEST conformational analysis method. As an exception, (−)-meptazinol and its carbamate derivatives were calculated by both BEST and CAESAR conformation algorithms. Chemical features including hydrogen bond acceptor (HBA), ring aromatic (RA), positive ionizable (PI) and hydrophobic (HYD) features were selected and each feature was given parameters from a minimum of 1 to a maximum of 5. The minimum interfeature distance was set as a value of 2.50 and the maximum excluded volumes was set to 5. Different weights were assigned to the features and weight variation was the default value of 0.302. Otherwise default parameters were used.

Chemistry

General

All reagents except phenyl isocyanate were of commercial quality. Phenyl isocyanate was prepared from aniline and bis(trichloromethyl) carbonate. Rivastigmine hydrochloride standard was available from Sunve (Shanghai) Pharmaceutical Co., Ltd. Melting points were measured in open glass capillary tubes with Thiele-Dennis tube, and were uncorrected. Specific rotation ([α]D) was determined on a JASCO-1020 rotatory apparatus. IR data were recorded on an AVATAR 360FT-IR spectrometer (KBr). NMR data were recorded on a Mercury Plus 400 instrument. Chemical shifts (δ) are expressed in parts per million (ppm), relative to tetramethylsilane (TMS) as an internal standard. Signals of active hydrogen disappeared after D_2O exchange. Mass spectrum was measured on an Agilent 1100 Series LC/MSD 1946D spectrometer. Elemental analysis was tested on vario EL III element analyzer. Purity of the target compound was verified via HPLC. The elution with acetoni-trile-0.01 mol/L KH_2PO_4 (pH =4.0) (33:67) was running through a Diamonsil C18(2) (200 × 4.6 mm, 5 μm) column at a flow rate of 1.0 mL/min and at the temperature of 30 °C using UV detection at 233 nm.

Synthesis of (S)-3-(3-ethyl-1-methylazepan-3-yl)phenyl dimethylcarbamate (42)

To a cooled and stirred mixture of 80% sodium hydride (0.15 g, 5.00 mmol) in 10 mL dry tetrahydrofuran, a solution of (−)-meptazinol (0.40 g, 1.71 mmol) in 10 mL dry tetrahydrofuran was added dropwise. The mixture was stirred in ice-water bath for 30 min, then N,N-dimethylcarbamoyl chloride (195 μL, 2.06 mmol) was added. After stirring at room temperature for 2 h, solvents were removed under reduced pressure. Then, water (20 mL) was added, and the mixture was extracted with EtOAc (15 mL × 2). Combined
Synthesis of (S)-3-(3-ethyl-1-methylazepan-3-yl)phenylcarbamate (43)

(−)-Meptazinol (0.40 g, 1.71 mmol) was dissolved in anhydrous ether (15 mL), and a piece of Na metal (approximately 5 mg) was added. The reaction mixture was stirred at room temperature for 3 h till the starting material had disappeared. 5 mL of H2O were added. The reaction mixture was stirred at room temperature for 10 min, then phenyl isocyanate (233 μL, 2.13 mmol) was added. The reaction mixture was stirred at room temperature for 3 h till the starting material had disappeared. 5 mL of H2O were added to destroy any trace of remaining unreacted phenylisocyanate and pH was adjusted to 3 by adding 1N HCl. The mixture was washed with ether (10 mL × 3), basified with saturated Na2CO3 aqueous solution (adjusting pH to 9), and then extracted with ether (10 mL). The latter ether layer was washed with brine, dried over anhydrous Na2SO4, and concentrated in vacuo to give a whitish oil (0.39 g, 82% yield): mp 122–123°C; m/z 353.2 [M + H]+; Anal. C22H28N2O2·HCl·1/4H2O (C, H, N).

Determination of the enzyme kinetic parameters Km and Vmax

10 μL inhibitors of different concentrations and 50 μL rHuAChE enzyme solution of 0.5 U/mL were mixed and incubated for 20 min, then 75 μL of DTNB solution and 100 μL of ATCh solution (concentration ranging from 0.057 mM to 0.2 mM) were added to the mixture. Enzyme activity was determined right after ATCh was added by modified Ellman’s spectrophotometrical method. The Km and Vmax values for AChE inhibition were calculated by regression analysis of Lineweaver–Burk plots (1/velocity versus 1/[substrate]).

Determination of carbamoylation and decarbamoylation rate constants

The EDA CIM disk was first connected to a syringe pump and equilibrated with 10 column volumes (CV) of triethylamine 50 mM aqueous solution and 10 CVs of 0.5 M Na2CO3 aqueous solution (adjusting pH to 9), and then extracted with ether (10 mL × 3). The latter ether layer was washed with brine, dried over anhydrous Na2SO4, and filtered to obtain a clear ether solution of the product. Evaporation of the solvent gave 43 (0.43 g, 72% yield) as a white solid. Acidification of 43 in dry ether using HCl-ether (adjusting pH to 4) afforded 43 hydrochloride as a white powder (0.39 g, 82% yield): mp 122–127°C; ν [CHCl3] = 1894 cm−1 (C = 0.108, MeOH); 1H NMR (400 MHz, DMSO-d6, TMS): δ = 10.23 (s, H, CONH), 9.98 (br s, 1/2H, NH+), 8.59 (br s, 1/2H, NH+), 7.50–7.40 (m, 3H, Ar-H), 7.33–7.21 (m, 3.5H, ArH), 7.16–7.10 (m, 1.5H, ArH), 7.04 (t, J = 7.43 Hz, H, Ar-H), 4.00–3.60 (m, 5H, N–CH3), 3.50–3.40 (m, H, N–CH2), 3.11 (m, 2H, N–CH2), 2.84 (m, 3H, N–CH3), 2.19–1.43 (m, 8H, CH2), 0.51 ppm (t, J = 7.04 Hz, 3H, CH3); 13C NMR (DMSO-d6–d3): 151.7 (C = O), 150.9 & 150.7 (C≡N), 145.5 & 144.0 (C=N), 138.6 (C≡N), 129.6 & 129.4 (C≡N), 128.9 (2 C≡N), 123.9 & 123.4 (C≡N), 123.0 (C≡N), 120.6 (C≡N), 120.3 (C≡N), 120.2 (C≡N), 118.5 (C≡N), 66.2 & 62.9 (NCH2), 59.5 & 58.0 (NCH2), 47.1 & 46.4 (NCH3), 44.2 & 43.9 (C), 35.9 & 35.4 (CH2), 33.5 & 33.0 (CH3), 26.3 & 24.8 (CH2), 20.6 & 20.4 (CH2), 8.2 & 8.0 (CH3); MS (ESI): m/z 353.2 [M + H]+; Anal. C18H28N2O2·HCl·1/4H2O (C, H, N). HPLC: tR = 8.5 min, 98.4% purity.

In vitro AChE/BChE inhibition assays

Inhibitory activities of the compounds toward AChE and BChE were evaluated by Ellman’s method, employing mice brain homogenate as source of AChE and mice serum as source of BChE. Briefly, 270 μL of a solution of AChE (1:9 w/v homogenate in 0.1 M phosphate buffer (PB), pH 7.4) and 30 μL of a solution of the tested compound (42, 43, or rivastigmine, six to seven concentrations) were mixed adequately. After incubation for 20 min at 37°C, Ellman’s reagent (300 μL, 5.5’-dithiobis(2-nitrobenzoic acid) (DTNB), 0.5 mM in 0.1 M PB, pH 7.4) and acetylthiocholine iodide (ATCh) (300 μL of 0.5 mM water solution) were added successively, and percent inhibition was determined by absorbance changes at 412 nm detected by UV spectrophotometry compared with control. BChE inhibition assay was similarly carried out using butrylthiocholine iodide (BTCh) (0.5 mM) as the substrate and BChE (1:19 v/v serum in 0.1 M PB, pH 7.4) as the enzyme source. The concentration of a compound that produced 50% inhibition of the enzyme activity, namely IC50 value, was calculated by nonlinear least squares regression of the response-concentration (log) curve. Results are reported as the mean ± SEM (standard error of the mean) of IC50 obtained from at least three independent measures.

\[ K_D = \frac{k_i}{k_s} \] (1)
Quantification of APP by high content screening (HCS cellomics)

Human neuroblastoma SH-SY5Y cells transfected with wild-type APP695 gene (SH-SY5Y-APP695) of 5 × 10^4/well (SH-SY5Y-APP695 kindly provided by Prof. Shengdi Chen, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine) were plated in 200 μL culture medium (DMEM/F12, 10% Fetal Bovine Serum, 300 μg/mL G418, 100 U/mL penicillin + 100 μg/mL streptomycin) and incubated for 48 h at 37°C in 5% CO2. Compounds 42 and 43 dissolved in culture medium were applied to the cells and incubated by Bliss method.

Quantification of Aβ40/Aβ42 by ELISA

The cell culture medium was collected and added with phenylmethylsulfonyl fluoride (PMSF) at the final concentration of 0.1%. The cell culture medium was collected and added with phenylmethylsulfonyl fluoride (PMSF) to the final concentration of 0.1%. The cell culture medium was collected and added with phenylmethylsulfonyl fluoride (PMSF) and incubated for 48 h at 37°C in 5% CO2 for 16 h. The cells were fixed and permeabilized. Mouse anti-APP monoclonal antibody N-terminus (MAB348, CHEMICON) and Goat anti-Mouse IgG conjugated to Alexa Fluor 555 were used as primary antibody and secondary antibody to detect APP while Hoechst Dye was used to stain nucleus simultaneously. KineticScan HCS System was employed to automatically find, focus, image, and analyze the cells on double-fluorescence channel guided by Target Activation Bioapplication. Fluorescence Unit of APP versus that of nucleus was used to evaluate the expression of APP, in order to balance the background.

Acute toxicity test

Acute toxicity was evaluated in mice of both sexes (20–25 g Kunming mice from experimental animal center of Shanghai Jiao Tong University School of Medicine). All animals were housed in plastic cages with food and water ad libitum and maintained on a 12/12 h light/dark cycle at 22 ± 1°C. They were randomly assigned to one of the five concentrations between 0% and 100% lethal rate according to our preliminary studies (data not shown). Compounds 42 and 43 were dissolved in a 1:10 propylene glycol-normal saline mixture at 0.1 mol/L and then diluted to the final stepwise concentrations with normal saline. Each concentration of the compound was orally or intraperitoneally administered to a group of 10 animals. After two weeks of observation, the lethal rate for each group was measured. A 95% confidence interval for 42 or 43 after oral or intraperitoneal administration was calculated by Bliss method.

Results and discussion

3D pharmacophore generation

To find out common structural elements necessary for AChE inhibition, quantitative 3D pharmacophore modeling was performed in silico using Discovery Studio v2.5 (DS, Accelrys, San Diego, CA) on 25 carbamate-type AChE inhibitors with diverse scaffolds. Carbamates with comparable IC50s tested by Ellman’s method and using physostigmine or rivastigmine as positive control were collected from the literature. As illustrated in Table 1, different phoshystigmine derivatives (A), enantiomers (B), and 8-carba analogs (C) had been enrolled into the training set (1–11). Rivastigmine derivatives (D) and conformationally restricted closed-ring rivastigmine analogs with benzopyrano[4,3-b]pyrrole (E), aminoindane (F) and aminotetralin (G) scaffolds had also been included (12–23). Phenol compounds without carbamoyl group, such as (−)-eseroline (24) and (25) fell into the category of inactive compounds. Similar values of all the training set compounds covered a range of three to four orders of magnitude, spanning from 8 nM to 40 μM (Table 1).

3D QSAR Pharmacophore Generation module was used to build pharmacophore models based on HBA, RA, PI and HYD features. Top 10 resultant pharmacophore hypotheses were generated and statistical parameters were used to select the best pharmacophore model. The best pharmacophore model should have the highest cost difference, lowest root mean square (RMS), and best correlation coefficient. Fixed and null costs are two important theoretical values to evaluate pharmacophore hypotheses, and the difference between null and fixed cost (cost difference) represents the goodness of a pharmacophore model. A cost difference of 40–60 means a predictable correlation probability of 75–90%. Correlation coefficient is based on linear regression of experimental versus estimated activities. In this study, top-ranked hypothesis was selected as the best pharmacophore model due to the highest cost difference (Δcost: 46.16), lowest RMS (RMS: 0.78), and best correlation coefficient (r: 0.95) (Table 1).

Experimental and estimated activities, fit values, and corresponding error values of the training set molecules are listed in Table 1. Error is the ratio between estimated activity and experimental activity. Estimated activity is predicted based on fit value. Fit value indicates how exactly structural components in a molecule are localized in the center of pharmacophoric feature spheres, and thus represents how well a molecular conformation matches the pharmacophore model. All the training set compounds were predicted in their same order of magnitude except that compound 21 was underestimated with an error of +10. The most active compound 15 (IC50 8 nM) had a fit value of 9.20, whereas the least active compound 25 (IC50 40 000 nM) showed lesser value of 5.55.

The best pharmacophore model consists of five chemical features (HBA, RA, PI, and two HYD features) and two excluded volumes (Figure 2(a,b)). A pair of green spheres indicates one HBA feature with an arrow showing the direction of hydrogen bond. A pair of orange spheres indicates one RA feature with an arrow showing a normal to the aromatic plane. Red sphere stands for PI feature, and cyan ones are indicative of two HYD regions. Gray spheres represent two excluded volumes that unfavorable steric effects may occur. Spatial disposition of the model features was described in Figure 2(a) and distances between feature centers were labeled in Figure 2(b). Figure 2(c–f) illustrated the mapping of representative training set compounds to the pharmacophore model.
Features in the pharmacophore model were assigned different weights, which indicated varied importance. HBA (weight: 3.18) is inevitably the most important feature since carbamoyl group is the basis of covalent carbamylation. Thus phenol compound 25 (Figure 2(d)), which failed to fit the HBA feature, showed very low activity; whereas corresponding methylcarbamate 15 (Figure 2(c)) was the most active compound. PI and RA (weight: 1.99) were mapped to amino and phenyl groups, respectively. Protonated amine interacts with aromatic residues in the catalytic site via cation-π interactions, which stabilizes the transition state of inhibitor-enzyme conjugate in the process of carbamylation. The distance between HBA and PI, namely O-N distance between the oxygen of

Table 1. Structures of training set molecules (1–25), their experimental IC₅₀’s reported in the literature, and estimated IC₅₀’s based on fit values to the pharmacophore model.a

| Cpd. | Isomer/Posb | R¹ | R² | R³ | R⁴/X | R⁵ | Experimental IC₅₀ (nM)c | Estimated IC₅₀ (nM) | Fit value | Errord | Referencee |
|------|------------|----|----|----|------|----|------------------------|---------------------|-----------|--------|------------|
| 1    | (−)        | Me | H  | Me | Me  | Me | 28                     | 23                  | 8.75      | −1.2   | 22        |
| 2    | (+)        | Me | H  | Me | Me  | Me | 9900                   | 2000                | 6.80      | −4.8   | 22        |
| 3    | (−)        | Me | H  | Me | H   | H  | 11                     | 21                  | 8.79      | +1.9   | 22        |
| 4    | (−)        | Ph | H  | Me | H   | H  | 1500                   | 1300                | 6.99      | −1.1   | 22        |
| 5    | (−)        | Ph | H  | Me | Me  | Me | 24                     | 19                  | 8.83      | −1.3   | 22        |
| 6    | (+)        | Ph | H  | Me | Me  | Me | 3500                   | 5100                | 6.40      | +1.5   | 22        |
| 7    | (+)        | 2’/Me-Ph| H | Me | Me  | Me | 10                     | 17                  | 8.88      | +1.7   | 23        |
| 8    | (+)        | 2’/Me-Ph| H | Me | Me  | Me | 5500                   | 1500                | 6.92      | −3.6   | 24        |
| 9    | (−)        | Me | H  | Ph | Me  | Me | 9300                   | 3100                | 6.62      | −3.0   | 24        |
| 10   | (−)        | Me | H  | Me | Et  |    | 38                     | 31                  | 8.61      | −1.2   | 26        |
| 11   | (−)        | Me | H  | H  | Et  |    | 250                    | 320                 | 7.60      | +1.3   | 26        |
| 12   | Me         |     | H  |    |     |    | 13²                    | 63                  | 8.31      | −4.8   | 27        |
| 13   | Me         |     |     |    |     |    | 78⁴                    | 93                  | 8.14      | +3.5   | 27        |
| 14   | Me         |     |     |    |     |    | 3000⁵                  | 1900                | 6.83      | −1.6   | 27        |
| 15   | 6−         | Me | H  |     | S   |    | 8                      | 8                   | 9.20      | 1.0    | 28        |
| 16   | 6−         | Me | H  |     | CH₂ |    | 17                     | 13                  | 8.99      | −1.3   | 28        |
| 17   | 6−         | Me | H  |     | O   |    | 30                     | 12                  | 9.02      | −2.5   | 28        |
| 18   | 7−         | Me | H  |     | O   |    | 1900                   | 1600                | 6.91      | −1.2   | 28        |
| 19   | 8−         | Me | H  |     | O   |    | 16000                  | 5700                | 6.35      | −2.9   | 28        |
| 20   | 5−         | Me | Me |     |     |    | 760                    | 1300                | 7.01      | +1.7   | 29        |
| 21   | 4−         | Me | Me |     |     |    | 460                    | 4600                | 6.44      | +10    | 29        |
| 22   | 8−         | Me | Me |     |     |    | 1500                   | 1600                | 6.91      | +1.9   | 29        |
| 23   | 7−         | Me | Me |     |     |    | 3200                   | 4900                | 6.41      | +1.5   | 29        |
| 24   | (−)        | Me | Me |     | >10000 | 12000| 6.04      | +1.2    | 29        |
| 25   | (−)        | Me | Me |     | 40000 | 36000| 5.55      | −1.1    | 28        |

aHypo cost = 116.65, Fixed cost = 108.67, Null cost = 162.82. Statistic parameters of the model and the training set: cost difference (Δcost = 46.16); root mean square (RMS = 0.78; correlation coefficient (r = 0.95).
bPosition where carbamoyloxyl groups substituted.
cAChE from human erythrocytes was used unless otherwise indicated.
dRatio between estimated and experimental IC₅₀ values. “+” indicates that the estimated IC₅₀ is higher than the experimental IC₅₀; “−” indicates that the estimated IC₅₀ is lower than the experimental IC₅₀; a value of 1 indicates that the estimated IC₅₀ is equal to the experimental IC₅₀.
eReferences to the literatures that reported the experimental AChE inhibitory activities.
fIn this special case, mice brain AChE was used.
carbonyl and the nitrogen of amine, plays an important role in the inhibition of AChE for carbamate-type compounds. As Figure 2(b) illustrated, the optimal distance between HBA and PI was 8.281 Å, indicating a range from 7.281 to 9.281 Å.

Unexpectedly, a small but important region of HYD (weight: 2.58) was identified in the model very near to the PI and RA features, which explained the activity difference between enantiomers. Figure 2(e) showed the alignment of phosophystigmine (1) to the pharmacophore model. Carbamoyl group and phenol ring overlapped to HBA and RA features, respectively. Nitrogen atom in 1-position instead of 8-position was aligned to the PI feature. (3aS)-methyl group in (3aS)-physostigmine (1) perfectly matched the HYD region (Figure 2(e)) and thus it shows high potency (IC₅₀ 28 nM). In contrast, the R-enantiomer (3aR)-physostigmine (2) is much less potent (IC₅₀ 9900 nM). It demonstrated that configurational inversion of the two asymmetric centers (3a and 8a) caused crucial conformational changes which led to pharmacophore model mismatch. When (3aS)-physostigmine (1) yielded to map into the HYD region as shown in Figure 2(f), its phenyl group switched away from the RA feature.

The proposed pharmacophore model was then validated by a test set of 16 compounds. Estimated AChE inhibitory activities based on fit values are listed in Table 2. All the test set compounds were predicted in their same order of magnitude. Consequent correlation coefficient of 0.91 for the test set indicated good predictive power of the pharmacophore model.

**Mapping of the pharmacophore model into AChE active site**

To extend our knowledge of the pharmacophore model from ligand basis to its interactive target, all model features were fitted into AChE active site. A recent study on the X-ray crystal structure of a complex of (−)-bisnorcymserine (Figure 3(a)) and AChE (PDB code 3ZV7) had revealed that a leaving group, (−)-bisnoreseroline (Figure 3(a)), was trapped in the catalytic site. The binding pose of (−)-bisnorphysostigmine (3) was supposed similar to that of (−)-bisnorcymserine since they had a common leaving group. Atom coordinates of the crystal AChE structure were fitted to those of the pharmacophore model through heavy atom superposition of crystal (−)-bisnoreseroline structure upon pharmacophoric (−)-bisnorphysostigmine (3) conformation (Figure 3(b)). Figure 3(c) showed mapping of the pharmacophore model into the active site of AChE. Residues involved in inhibitor-enzyme interactions were represented as gray lines. Interestingly, two
Table 2. Estimated $IC_{50}$s of the test set compounds (26–41) compared with their experimental $IC_{50}$s reported in the literature to validate the pharmacophore model.\(^a\)

| Cpd. | R\(^1\) | R\(^2\) | R\(^3\) | R\(^4\) | R\(^5\) | Experimental $IC_{50}$ (nM)\(^b\) | Estimated $IC_{50}$ (nM) | Fit value | Error\(^c\) | Reference\(^d\) |
|------|--------|--------|--------|--------|--------|-------------------------------|----------------|-----------|-----------|-------------|
| 26   | Me     | H      | Me     | Me     | H      | 57                           | 20             | 8.80      | −2.9      | 30          |
| 27   | Me     | H      | Me     | H      | Me     | 2200                         | 480            | 7.43      | −4.6      | 30          |
| 28   | Me     | H      | Me     | H      | Me     | 21                           | 22             | 8.76      | +1.0      | 22          |
| 29   | Me     | H      | Me     | H      | Me     | 190                          | 760            | 7.23      | +4.0      | 31          |
| 30   | Ph     | H      | Me     | Me     | H      | 41                           | 18             | 8.86      | −2.3      | 30          |
| 31   | Ph     | H      | Me     | Me     | H      | 5700                         | 2900           | 6.64      | −2.0      | 30          |
| 32   | 2’Et-Ph| Me     | H      | Me     | H      | 10                           | 24             | 8.73      | +2.4      | 23          |
| 33   | 2’iPr-Ph| Me    | H      | Me     | Me     | 15                           | 26             | 8.69      | +1.7      | 24          |
| 34   | Ph     | H      | Ph     | Me     | Me     | 10 000                       | 4600           | 6.45      | −2.2      | 26          |
| 35   | Me     | H      | Me     | Pr     | H      | 150                          | 68             | 8.28      | −2.2      | 28          |
| 36   | Me     | H      | H      | Pr     | H      | 2000                         | 960            | 7.12      | −2.1      | 26          |
| 37   | 75     | H      | Me     | H      | H      | 74                           | 160            | 7.89      | +2.2      | 28          |
| 38   | 7      | H      | Me     | H      | H      | 75                           | 23             | 8.74      | +3.3      | 32          |
| 39   | 680\(^e\) | H    | Me     | H      | H      | 150                          | 68             | 8.28      | −2.2      | 33          |
| 40   | 1200\(^f\) | H   | Me     | H      | H      | 2000                         | 960            | 7.12      | −2.1      | 26          |
| 41   | 2200   | H      | Me     | H      | H      | 2200                         | 630            | 7.31      | −3.5      | 35          |

\(^a\) Statistical parameters of the test set: correlation coefficient ($r = 0.91$).

\(^b\) AChE from human erythrocytes was used unless otherwise indicated.

\(^c\) Ratio between estimated and experimental $IC_{50}$ values. “$-$” indicates that the estimated $IC_{50}$ is higher than the experimental $IC_{50}$; “$+$” indicates that the estimated $IC_{50}$ is lower than the experimental $IC_{50}$; a value of 1 indicates that the estimated $IC_{50}$ is equal to the experimental $IC_{50}$.

\(^d\) References to the literatures that reported the experimental AChE inhibitory activities.

\(^e\) Mice brain AChE was used.

\(^f\) AChE from bovine erythrocytes was used.

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Figure 3. (a) Structures of (−)-bisnorycmserine and (−)-bisnoreseroline; (b) Superimposition of the crystal (−)-bisnoreseroline structure (light green, in big-ball-and-stick) on the pharmacophoric (−)-bisnorphysostigmine (3, magenta, in ball-and-stick) conformation; (c) The pharmacophore model fitted into the active site of AChE (PDB code 3ZV7). (Pharmacophoric features: green, HBA; orange, RA; red, PI; cyan, HYD; gray, Xvol; Active site residues were shown as gray lines; Key residues mapped to the excluded volumes were highlighted as yellow sticks.)
Although built on azapane scaffold, in azapane ring of 43 important PI (red) center and HYD (cyan) region. The only nitrogen features in the model just as phenserine did, especially at the very 

Figure 3(b,c). HBA was located nearby the hydroxyl of Ser200, triad residues, Ser200 and His440, highlighted as yellow sticks in Figure 4(a) and (c) and (Figure 4(b)), and the spatial overlapping of phenserine (Figure 4(a)) and (Figure 4(b)). HBA; orange, RA; red, PI; cyan, HYD; gray, Xvol.

Figure 4. 2D structures of phenserine (a) and (−)-meptazinol phenylcarbamate (b) with color backgrounds highlighting common chemical features, and 3D overlays of phenserine (c, yellow, in ball-and-stick) and (−)-meptazinol phenylcarbamate (d, green, in stick) with the best pharmacophore model. (Pharmacophoric features: green, HBA; orange, RA; red, PI; cyan, HYD; gray, Xvol.)

Table 3. Predicted AChE inhibitory activities of 42, 43 and (−)-meptazinol by BEST and CAESAR algorithms.

| Compounds       | BEST algorithm | CAESAR algorithm |
|-----------------|----------------|------------------|
|                 | Estimated IC50 (nM) | Fit value | Estimated IC50 (nM) | Fit value |
| 42              | 100            | 8.10             | 75                | 8.23 |
| 43              | 560            | 7.36             | 370               | 7.54 |
| (−)-Meptazinol  | 3300           | 6.59             | 18 000            | 5.86 |

Phenserine 24.0 ± 6.0 g 1300 ± 85g 54 25 (i.p.) iSee reference55. p.o.: oral administration; i.p.: intraperitoneal administration.

Table 4. AChE and BChE inhibitory activities, selectivity and acute toxicity of (−)-meptazinol carbamates, compared with classical carbamate-type AChE inhibitors.

| Compounds                  | AChE IC50 ± SEM (nM) | BChE IC50 ± SEM (nM) | Selectivity for AChE | LD50 (mg/kg) |
|---------------------------|----------------------|----------------------|----------------------|---------------|
| 42                        | 6.93 ± 2.45          | 3.17 ± 1.34          | 2.1                  | 73 (p.o.); 45 (i.p.) |
| 43                        | 31.6 ± 3.5           | 67.1 ± 23.7          | 0.46                 | N/A<sup>e</sup> |
| (−)-Meptazinol            | 41 000 ± 14 000<sup>f</sup> | 15 000 ± 4000<sup>f</sup> | 3.1                 | 3-6 (p.o.)<sup>g</sup> |
| Rivastigmine              | 5460 ± 1470          | 1590 ± 38            | 0.29                 | 4.5 (p.o.)<sup>h</sup> |
| Physostigmine             | 27.9 ± 2.4<sup>i</sup> | 16.0 ± 2.9<sup>j</sup> | 1.72                | 4.5 (p.o.)<sup>h</sup> |
| Phenserine                | 24.0 ± 6.0<sup>k</sup> | 1300 ± 85<sup>k</sup> | 3.1                 | 25 (i.p.)<sup>l</sup> |

<sup>a</sup>Mice brain homogenate was the source of AChE unless otherwise indicated.
<sup>b</sup>Mice serum was the source of BChE unless otherwise indicated.
<sup>c</sup>Selectivity for AChE: IC50 for BChE divided by IC50 for AChE.
<sup>d</sup>See reference17.
<sup>e</sup>N/A: not available.
<sup>f</sup>See reference34.
<sup>g</sup>Human erythrocyte AChE and human serum BChE were used, see reference22.
<sup>h</sup>See reference34.
<sup>i</sup>See reference17. p.o.: oral administration; i.p.: intraperitoneal administration.

**Pharmacophore-based design and synthesis**

As we early reported, (−)-meptazinol is a moderate AChE inhibitor (IC50 41 μM<sup>17</sup>, Table 4) and it binds the enzyme by reversible mechanism. Guided by the pharmacophore model generated above, we selected (−)-meptazinol as the scaffold to build carbamoyl groups on. (−)-Meptazinol dimethylcarbamate (42) and phenylcarbamate (43) (Figure 1) were designed by carbamoylating the phenolic hydroxyl of (−)-meptazinol. They were supposed to be good AChE inhibitors for their perfect superimposition upon the model.

Figure 4 showed the geometrical structural similarity between phenserine (Figure 4(a)) and (−)-meptazinol phenylcarbamate (43) (Figure 4(b)), and the spatial overlapping of phenserine (Figure 4(c)) and 43 (Figure 4(d)) with the pharmacophore model. Although built on azapane scaffold, 43 matched four of the five features in the model just as phenserine did, especially at the very important PI (red) center and HYD (cyan) region. The only nitrogen in azapane ring of 43 resembled the N1-nitrogen of phenserine.
Experimental activity. By CAESAR algorithm, the estimated IC$_{50}$ values of 42 and 43 were predicted as hydrochloride salts for the following in vitro assays. Structures of the hydrochloride salts were characterized by $[\alpha]_D$, IR, $^1$H NMR, $^{13}$C NMR, MS, and elemental analysis.

**Cholinesterase inhibitory potency and selectivity**

The synthesized carbamate compounds 42 and 43 were tested in vitro for AChE/BChE inhibitory potency and selectivity (Table 4). Mice brain homogenate and mice serum were used as sources of AChE and BChE, respectively. The dimethylcarbamate 42 inhibited AChE with IC$_{50}$ value of 6.93 nM, about 790 times lower than that of rivastigmine (IC$_{50}$ 5460 nM). The phenylcarbamate 43, showing an IC$_{50}$ of 31.6 nM, was about 170 times more potent than rivastigmine and almost five times less potent than 42. Compared with the parent compound (-)-meptazinol, 42 and 43 showed a 5900-fold and 1300-fold increase, respectively, in the inhibition of mice brain AChE. With regard to activities reported by Yu et al.\textsuperscript{22}, 42 was four times more potent than phystosginine (IC$_{50}$ 27.9 nM), while 43 was 1.3 times less potent than phenerserine (IC$_{50}$ 24.0 nM).

As for selectivity, 42 was slightly more selective (twofold) to BChE similar to rivastigmine and phystosginine, while 43 was an AChE-selective inhibitor, showing a twofold selectivity for AChE versus BChE (IC$_{50}$ 67.1 nM). Similar to phenerserine, 43 would have less peripheral side effects and lower acute toxicity than those BChE-selective carbamates, such as phystosginine and 42.

![Scheme 2. Synthesis of 42 and 43.\textsuperscript{a}](image)

\textsuperscript{a}Reagents and conditions: (i) Me$_2$NCOCl, NaH, dry THF, 0 °C to rt, 2 h, 100%; (ii) PhNCO, Na, dry Et$_2$O, rt, 3 h, 72%.

**Table 5.** $K_m$ and $V_{max}$ values of 42 and 43 on rHuAChE.

| Compounds | Concentration (nM) | $K_{m}$ (µM) | $V_{max}$ (µM/min) |
|-----------|-------------------|--------------|-------------------|
| 42        | 0                 | 112.67       | 34.94             |
|           | 100               | 113.97       | 28.73             |
|           | 250               | 115.10       | 24.70             |
| 43        | 0                 | 147.11       | 19.92             |
|           | 25                | 145.87       | 17.84             |
|           | 50                | 143.90       | 14.94             |

**Table 6.** Kinetic constants of 42 and 43 on rHuAChE (Mean ± SD, $n = 6$).

| Compounds | $k_0$ (M$^{-1}$) | $k_i$ (M$^{-1}$min$^{-1}$) | $k_e$ (min$^{-1}$) |
|-----------|-----------------|--------------------------|-------------------|
| 42        | $(3.02 ± 0.51) \times 10^{-6}$ | $(4.47 ± 1.21) \times 10^{-5}$ | $(1.35 ± 0.31) \times 10^{-2}$ |
| 43        | $(1.08 ± 0.30) \times 10^{-7}$ | $(2.12 ± 0.46) \times 10^{-5}$ | $(2.18 ± 0.12) \times 10^{-2}$ |

**Mechanism of enzyme inhibition and kinetic parameters**

Understanding of potent AChE inhibitors’ mechanism of action and kinetic parameters is key information to establish the structure-activity relationship and design new compounds for the treatment of AD. The characteristics of AChE activity inhibition by 42 and 43 were revealed by enzyme kinetics assays. The plots of residual enzyme activity versus enzyme concentration at different concentrations of 42 and 43 gave a family of straight lines with a y-axis intercept, suggesting that both compounds were reversible AChE inhibitors. Their enzyme inhibitory properties were further modeled using double-reciprocal plots. Variance of the velocity of control group could be explained as the degradation of enzyme. Increasing the concentrations of 42 and 43 led to a decrease in $V_{max}$ and an unvaried $K_m$ (y-intercepts) (Table 5). 42 decreased the $V_{max}$ by 18% and 29% at the concentrations of 100 and 250 nM and 43 decreased the $V_{max}$ by 10% and 25% at the concentrations of 25 and 50 nM, consistent with the typical characteristics of uncompetitive inhibitors. The observed results showed that both inhibitors bound only to enzyme-substrate complex, not the free enzyme.

The inhibition of AChE by carbamates involves carbamoylation of the enzyme and production of a covalent adduct. The carbamoylated enzyme is then hydrolyzed to regenerate the free enzyme. The process is time-dependent, therefore, determination of the kinetic parameters is of utmost importance to assess time of action. Bartolini et al.\textsuperscript{41} has reported that AChE immobilized disk, which could maintain enzymatic activity for about 2 months, was a powerful tool to evaluate both the carbamoylation and the decarbamoylation constants in single experiment. In this study, the well-known pseudo-irreversible AChE inhibitor phystosginine (1) was first selected as a reference compound to verify the reliability of our disk. Percent inhibition of enzyme activity [(A$_0$–A)/A$_0$ $\times$ 100%] was plotted versus time. The curve was fitted to Perola’s mathematical equation. The calculated $k_i$, $k_e$, and $K_D$ of phystosginine were $(4.78 ± 1.13) \times 10^{-5}$, $1.94 ± 0.36$ $\times 10^{-2}$ min$^{-1}$, and $4.09 ± 0.22$ $\times 10^{-8}$ M$^{-1}$, respectively, consistent with the reported values.
with the previously reported results\(^4\), indicating the AChE-immobilized disk we prepared was robust for the determination of kinetic constants of 42 and 43.

The data of the carbamoylation and decarbamoylation of AChE by 42 and 43 fitted well to Perola’s equation\(^4\). Figure 5 showed that the immobilized AChE in EDA CIM disk was time-dependently inactivated by 42 and 43 at 50 nM. The carbamoylation half-times of 42 and 43 were found to be 23.5 min and 20.3 min, respectively, longer than that of physostigmine (3.9 min) and rivastigmine (11.4 min) reported by Bartolini et al.\(^\text{39}\). About 2 h and 3 h flushing were required to achieve a complete recovery of AChE activity after complete inhibition by 42 and 43, respectively, similar to physostigmine (2 h) but much shorter than rivastigmine (34 h). The \(k_4\), \(k_5\) and \(k_7\) values of 42 and 43 are shown in Table 6. 43 could bind to and dissociated from AChE faster than 42, indicating that the enzyme was more affinitive to 42 than 43. And, 42 inhibited AChE more strongly than 43. There was no obvious difference of the \(k_1\) and \(k_3\) values between 42 and 43. The enzyme affinity, carbamoylation and decarbamoylation rates and the duration of the inhibition of 42 and 43 were similar to that of physostigmine, suggesting that both the compounds reacted with the enzyme as a pseudo-irreversible inhibitor, in a way typical to carbamates, through quick formation of an addition complex and subsequent slow decarbamoylation.

**Anti-amyloidogenic properties in SH-SYSY-APP\(_{695}\) cells**

Anti-amyloidogenic properties of 42 and 43 were evaluated with HCS in SH-SYSY-APP\(_{695}\) cells\(^3\). After exposure to 50 \(\mu\)M of 42 and 43 for 16 h, intracellular APP levels were markedly reduced by 23.5% and 26.9%, respectively (Figure 6(a)). Phenserine was reported to produce a 40% decrease of APP level at the same concentration\(^\text{51}\). At a lower concentration of 5 \(\mu\)M, 43 exhibited a 20.0% decrease of APP levels (Figure 6(a)).

Although APP over-expression is a risk factor to AD, APP is still necessary to maintain normal physiological function. So, it will be more important to evaluate anti-amyloidogenic properties by determining A\(\beta\) lowering effects, especially the neurotoxic form A\(\beta_{42}\). Application of 50 \(\mu\)M of 42 and 43 to SH-SYSY-APP\(_{695}\) cells remarkably reduced the level of A\(\beta_{42}\) by 34.0% and 51.9%, respectively (Figure 6(b)), keeping the level of less toxic A\(\beta_{40}\) unchanged (data not shown). The A\(\beta\) lowering effect of 43 was better than that of phenserine (31% decrease of total A\(\beta\)) at the concentration of 50 \(\mu\)M. Even at a lower concentration of 5 \(\mu\)M, 43 still produced a 30.5% decrease of the A\(\beta_{42}\) level (Figure 6(b)).

The actions of 42 and 43 on reducing APP and A\(\beta_{42}\) levels were very promising, but the mechanism was still complicated. Most AChE inhibitors produce A\(\beta\) lowering effects by altering APP into non-amyloidogenic pathway\(^\text{52}\). This anti-amyloidogenic property results from post-receptor effects, such as Protein Kinase C (PKC\(\alpha\)) activation, of the increased ACh level secondary to AChE inhibition. Exhibiting poorer AChE inhibition, 43 produced better APP and A\(\beta_{42}\) lowering properties in comparison with 42, which indicated additional non-cholinergic involvement in the anti-amyloidogenic effect of 43. Phenserine was reported to reduce the levels of APP and A\(\beta\) via a non-cholinergic mechanism by downregulating the translation of APP mRNA\(^\text{14}\). Although 43 was less potent than phenserine in reducing APP level, its ability to reduce A\(\beta\), especially the most neurotoxic A\(\beta_{42}\), was much higher than phenserine. It was possible yet still a hypothesis that 43 might have a direct action on the amyloidogenic processing pathway. Further experiments were still needed to clarify the mechanism.

**Acute toxicity**

The LD\(_{50}\) values of 42 and 43 were tested in mice after intraperitoneal (i.p.) and oral (p.o.) administration, and corresponding results are reported in Table 4. As the doses of 42 and 43 escalating, peripheral cholinergic side effects such as salivation, twitch, and incontinence were observed. 42 showed high acute toxicity (LD\(_{50}\) 1.4 mg/kg) after i.p. administration. When administered orally, 42 (LD\(_{50}\) 12 mg/kg) was almost three times less toxic than physostigmine (LD\(_{50}\) 4.5 mg/kg)\(^\text{53}\), although 42 showed four times higher potency than physostigmine in in vitro test. The LD\(_{50}\) of 43 (73 mg/kg, p.o.) was 12–24 times higher than that of rivastigmine (3–6 mg/kg, p.o.)\(^\text{54}\). If administered intraperitoneally, 43 (LD\(_{50}\) 45 mg/kg) was slightly less toxic compared with phenserine (25 mg/kg)\(^\text{55}\). Thus, 43 showed low acute toxicity and deserved further studies in cholinergic impairment animal models to evaluate its in vivo cognitive enhancement function.

**Conclusions**

In summary, (−)-meptazinol carbamate derivatives were designed based on a 3D pharmacophore model built using 3D QSAR Pharmacophore Generation module in DS from 25 known
carbamate-type AChE inhibitors. The best pharmacophore model consists of five chemical features (namely HBA, RA, PI, and two HYDs) and two excluded volumes. The existence of a HYD region near the PI feature have been recognized as essential chemical characteristics in the model to differentiate enantiomers. Merging of carbamoyl groups onto the (−)-meptazinol scaffold generated new bifunctional ligands with dual actions on both cholinesterase and amyloidogenic pathways.

The synthesized compounds 42 and 43 were verified as nanomolar cholinesterase inhibitors in in vitro assay. 42 and 43 showed uncompetitive inhibition and reacted with the enzyme as a pseudo-irreversible inhibitor, such as typical carbamates, through quick carbamoylation and subsequent slow decarbamoylation. 42 (IC50 6.93 nM) was more potent in inhibiting AChE than 43, and was slightly selective to BChE (two-fold). In acute toxicity test, 42 had lower LD50 values (12 mg/kg, p.o.) and showed more peripheral cholinergic side effects. However, the phenylcarbamate 43 was more promising and exhibited significant anti-cholinesterase and anti-amyloidogenic properties. 43 (IC50 31.6 nM) was 170 times more potent than rivastigmine in inhibiting AChE, and was 1.3 times less potent than phenserine. 43 exhibited a twofold selectivity for AChE, therefore milder peripheral side effects and lower acute toxicity were observed for 43 (LD50 73 mg/kg, p.o.). 43 also showed Aβ lowering effects (51.9% decrease of Aβ1-42) superior to phenserine (31% decrease of total Aβ) at the concentration of 50 μM. Even at a lower concentration of 5 μM, 43 still reduced APP level by 20.0% and reduced Aβ1-42 by 30.5%. The dual actions of cholinesterase inhibition and anti-amyloidogenesis indicated a potential use of 43 as symptomatic and disease-modifying agent for the treatment of AD, which deserved further studies in cholinergic impairment animal models.

Acknowledgements

We thank Professor Shengdi Chen, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, for providing the SH-SYSY-APP695 cells.

Disclosure statement

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Funding

This work was supported by the National Basic Research Program of China (973 Program, 2010CB529806); National Major Scientific and Technological Special Project for “Significant New Drugs Development” of Ministry of Science and Technology of China (2009ZX09103-077 and 2009ZX09301-011); National Natural Science Foundation of China (30772553, 30801393, 30801435, 30973509, 21202098, 81373395 and 81573415); Ph.D. Programs Foundation of Ministry of Education of China (200802461095); Science and Technology Commission of Shanghai Municipality (104131902700, 14431905600); and “Chen Guang” Project of Shanghai Municipal Education Commission and Shanghai Education Development Foundation (10CG03).

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