Novel acetylcholinesterase inhibitors: Synthesis and structure–activity relationships of phthalimide alkyloxyphenyl *N,N*-dimethylcarbamate derivatives

Qianfei Zhao^a, Guoquan Yang^b, Xiangdong Mei^b, Huizhu Yuan^b, Jun Ning^{b,*}

^a Research Center for Eco-Environmental Sciences, Academia Sinica, P.O. Box 2871, Beijing 100085, PR China

^b Key Laboratory of Pesticide Chemistry and Application, Ministry of Agriculture, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, PR China

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ABSTRACT

Based on the multiple binding sites of acetylcholinesterase (AChE), a series of AChE inhibitors: phthalimide alkyloxyphenyl *N,N*-dimethylcarbamate were designed and synthesized. AChE inhibitory activity and structure–activity relationship of the compounds were researched also. The influence of structural variations on the inhibitory potency was carefully investigated by modifying different alkyloxy chain length and position between phthalimide and phenyl *N,N*-dimethylcarbamate (PDM). The biological properties of the series were investigated by considering the activity on isolated enzyme. Some of the newly synthesized derivatives, when tested on isolated AChE from head of housefly (*Musca domestica*), were more active than PDM. The compounds **J1**, **J2** and **K1–K8** demonstrated higher inhibitory activity (5- to 404-fold) for AChE than that of PDM. In particular, compound **K1** displayed the best AChE inhibition (404-fold higher than PDM), which suggested that phthalimide group of **K1** strongly bound at the residues lining the gorge while phenyl *N,N*-dimethylcarbamate bound at the catalytic site.

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1. Introduction

On the basis of the structure of AChE, many potential dual-site binding inhibitors of AChE have been synthesized and demonstrated in drugs in order to treat or alleviate AD. Donepezil marketed as Aricept[®] was approved in 1996, which was the first drug for AD binding the two sites of AChE. E2020 had a unique orientation along the active-site gorge, extending from the anionic subsite of the active site, at the bottom, to the peripheral anionic site, at the top, via aromatic stacking interactions with conserved aromatic acid residues [1]. Compound **A** and **B** in Fig. 1 exhibited an excellent AChE inhibitory activity. The phthalimide ring occupied a similar position at the peripheral site and remained stacked onto the aromatic ring of Trp279 [2]. Compound **C** (*m* = 3) displayed the remarkable rat cortex AChE inhibition (190-fold higher than physostigmine). Compound **D** (*m* = 7) displayed AChE inhibition ability nearly equal to compound **C**. The tricyclic ring of compound **C** was not able to reach Trp279, but showed stacking interaction with Tyr330, Phe331 and Tyr121, which existed on the halfway of the gorge. The tricyclic ring of compound **D** reached Trp279 and bound Trp279 well [3,4]. Compound **E** and **F**, designed and synthesized as dual inhibitors of AChE and SERT, had a high affinity for AChE in the nanomolar range (compound **E** with

IC₅₀ = 14 nM, 785-fold higher than rivastigmine, and compound **F** with IC₅₀ = 36 nM, 305-fold higher than rivastigmine) [5].

Synthetic carbamate insecticides, which inhibited enzyme activity by covalently carbamylating the serine residue within the active-site gorge [6], have been used during the last several decades. Many carbamate insecticides such as metolcarb, methomyl, thiodicarb, carbofuran, carbosulfan and aldicarb have been widely used for control of insect pests in the world. Most carbamate used as insecticides were esters of aromatic and heterocyclic hydroxyl derivatives of *N*-methyl and *N,N*-dimethylcarbamic acid. There were no reports about phenyl *N,N*-dimethylcarbamate to be selected as the moieties to bind the catalytic site of dual or multiple-site inhibitors of AChE in pesticides. In order to obtain inhibitors able to bind at the catalytic site and the peripheral site or another sites on the halfway of AChE, we designed molecules composed by two moieties (phthalimide and *N,N*-dimethylcarbamate) for the binding at each sites and linked by different carbon number alkylene, synthesized a series of novel phthalimide alkyloxyphenyl *N,N*-dimethylcarbamates. Carbamates with protonated amine group in the middle of the chain were good in vivo AChE inhibitors but were incapable of reaching the sites of action in the insect, because the nerve fibers are protected by a lipophilic sheath [7], so the alkyloxy chains were selected as the linkers.

Housfly's AChE belonged to AChE 2 family, from fruitfly was taken as reference. The amino acids, including Ser, Glu, His of the active site and Trp of acetyl binding site, were identical between different species [8]. The AChE inhibitory ability of all the new

* Corresponding author. Fax: +86 10 62899142.

E-mail address: jning502@yahoo.com.cn (J. Ning).

Fig. 1. Structures of some dual-site binding inhibitors of AChE.

compounds was tested using the isolated AChE from head of housefly. An attempt to rationalize the structure–activity relationships of the series was carried out.

2. Materials and methods

2.1. Synthetic procedures

The ^1H NMR spectra were measured on a Bruker DPX300 NMR spectrometer with CDCl_3 as solvent. The following abbreviations were used to explain the multiplicities: s, singlet; d, doublet; t, triplet; m, multiplet. Analytical thin-layer chromatography (TLC) was performed on precoated plates (silica GF254) using UV light. Chromatographic separations were performed on silica gel (100–200 mesh) obtained from Qingdao Haiyang Chemical Co., Ltd., using flash column chromatography. Melting points were determined with a WRS-2A melting point apparatus.

2.2. General synthetic procedure for G1–G8

Compounds **G1–G8** were synthesized following the procedure previously reported [9] (Scheme 1). The reaction time was prolonged to 24 h, and the compounds were purified by silica gel column chromatography using petroleum ether/AcOEt (15:1–50:1).

2.3. General synthetic procedure for H1–H8 and I1–I8

Preparation of **H1–H8** and **I1–I8** was shown in Scheme 1. A stirred solution of **G** 10 mmol, catechol or resorcinol 25 mmol, and potas-

sium carbonate 30 mmol in dry acetone (50 ml) was refluxed for 24 h. The hot reaction mixture was filtered, and the solvent was condensed under reduced pressure. Then the condensed liquid was added into 5% brine with stirring to make the intermediates flocculate adequately. The solid was washed by water times and collected, dried under reduced pressure at 60 °C to give the crude product as a white or light yellow solid which were used directly in next reaction.

2.4. General synthetic procedure for J1–J8 and K1–K8

Preparation of **J1–J8** and **K1–K8** was shown in Scheme 1. Crude solid **H** or **I** ≈ 2.5 mmol and *N,N*-dimethylcarbamic chloride 3 mmol were dissolved in DMF (20 ml), then potassium carbonate (4.75 g, 30 mmol) was added. After the mixture was stirred for 8 h at room temperature, 5% brine (120 ml) was added to make the product flocculate adequately. The mixture was filtered, and the solid was washed with water twice, dissolved in AcOEt (20 ml). The liquid was washed with water (20 ml) three times, dried over Na_2SO_4 , filtered and evaporated under reduced pressure. The residue was purified by silica gel column chromatography using petroleum ether/AcOEt (1:1–3:1) as eluent mixtures of solvents in the proportions indicated for each case. The physical form, melting points and two-step yields of compounds **J1–J8** and **K1–K8** were listed in Table 1, and their ^1H NMR were listed in Table 2.

2.5. AChE inhibitory bioassay

To evaluate the biological profiles of these compounds, AChE inhibition was assayed in comparison with PDM.

Scheme 1. Synthesis of phthalimide alkyloxyphenyl *N,N*-dimethylcarbamates Regents and conditions: (i) dibromoalkane, acetone, reflux 24 h; (ii) resorcinol or pyrocatechol, potassium carbonate, acetone, reflux 24 h; (iii) dimethylcarbamic chloride, DMF, RT 8 h.

Table 1Appearance and two-step yields of compounds **J1–J8** and **K1–K8**.

Compd	Appearance	Two-step yields (%)
J1	Colorless viscous oil	65.2
J2	Colorless viscous oil	72.6
J3	Colorless viscous oil	72.7
J4	Colorless viscous oil	73.8
J5	Colorless viscous oil	62.5
J6	Colorless viscous oil	68.3
J7	White solid	60.4
(mp 64.2–64.5 °C)		
J8	Colorless viscous oil	62.2
K1	Colorless viscous oil	63.0
K2	Colorless viscous oil	68.6
K3	Colorless viscous oil	70.3
K4	Colorless viscous oil	64.5
K5	Colorless viscous oil	62.6
K6	Colorless viscous oil	62.2
K7	Colorless viscous oil	59.3
K8	Colorless viscous oil	58.1

2.6.1. Enzyme preparation

AChE was prepared from heads of adult housefly (female: male = 1:1) of susceptible strains, homogenized at 4 °C in ice-cold 0.02 M potassium phosphate buffer (pH 7.5), 2.5 heads/ml. The homogenate was centrifuged at 1000g for 15 min at 4 °C. Supernatants were transferred to fresh tubes and used as enzyme sources.

2.6.2. Inhibition of isolated AChE

AChE activity was determined by the method of Ellman et al. [10] using ATCh as substrate, with modifications described by Gorun [11]. After 0.1 ml inhibitor solution was incubated with 0.1 ml enzyme for 5 min at 30 °C, 0.1 mL ATCh (10 mM) was added, and the mixture was incubated for 15 min at 30 °C. The reaction was stopped when 3.6 mL DTNB (15 mM) solution contained 45% of ethanol was added. The absorbance at 412 nm was measured with UNICO UV-2102C. Four to six different concentrations of each compound were used in order to obtain inhibition of AChE activity ranging between 20% and 80%. Each concentration was analyzed

in triplicate. Assays were run with a blank containing all of the components except AChE in order to account for a nonenzymatic reaction. The reaction rates were compared and the percent inhibition due to the presence of test compounds was calculated. The percent inhibition of the enzyme activity due to the presence of increasing test compound concentration was calculated by the following expression: $100 - (v_i/v_o - 100)$, where v_i was the initial rate calculated in the presence of inhibitor and v_o is the enzyme activity. Inhibition curves were obtained for each compound by plotting the % inhibition vs the logarithm of inhibitor concentration in the assay solution. Parameters were determined for each curve and the IC_{50} values were extrapolated. The computer program used to analyze these data was SAS8.0 (SAS Institute Inc.).

3. Results and discussion

The inhibitory activity against AChE of *N,N*-dimethylcarbamate derivatives was reported in Table 3, expressed as IC_{50} values with PDM as the reference standard. From the IC_{50} values of compounds, some general conclusions about structure–activity relationships could be drawn from the results, and they were discussed also, as follows:

It appeared that the compounds **K1–K8** demonstrated higher inhibitory activity (5- to 404-fold) for AChE than for PDM, which might bind at the catalytic sites and another sites of the gorge (about 20 Å long and as narrow as 4.5 Å), besides the peripheral sites. Different length of phthalimide alkyoxyl, which might bind at the aromatic residues along the narrow gorge, were favorable for increasing the affinity of molecules to AChE while the substituent was in the *meta*-position.

The compounds having the same linker between phthalimide group and phenyl group at the *meta*-position were more potent than those substituted at *ortho*-position, which was different from the traditional carbamate insecticides [7,12,13]. The larger steric hindrance of *ortho*-alkyoxyl phthalimide than that of *meta*-position could cause the difference of activity from the traditional carbamate insecticides.

Table 2 1H NMR of compounds **J1–J8** and **K1–K8**.

Compd	δ (ppm)
J1	2.12–2.17 (m, 2H), 3.04 (s, 3H), 3.20 (s, 3H), 3.85–3.90 (t, 2H), 4.05–4.09 (t, 2H), 6.91–6.95 (t, 2H), 7.08–7.14 (m, 2H), 7.70–7.73 (m, 2H), 7.83–7.86 (m, 2H)
J2	1.77–2.04 (m, 4H), 2.98 (s, 3H), 3.12 (s, 3H), 3.73–3.78 (t, 2H), 4.00–4.03 (t, 2H), 6.86–6.90 (t, 2H), 7.05–7.15 (m, 2H), 7.70–7.81 (m, 2H), 7.81–7.85 (m, 2H)
J3	1.46–1.54 (m, 2H), 1.70–1.86 (m, 4H), 3.01 (s, 3H), 3.11 (s, 3H), 3.68–3.73 (t, 2H), 3.95–4.00 (t, 2H), 6.86–6.93 (t, 2H), 7.05–7.15 (m, 2H), 7.70–7.72 (m, 2H), 7.81–7.85 (m, 2H)
J4	1.37–1.54 (m, 4H), 1.65–1.80 (m, 4H), 3.00 (s, 3H), 3.11 (s, 3H), 3.67–3.72 (t, 2H), 3.94–3.98 (t, 2H), 6.87–6.92 (t, 2H), 7.05–7.15 (m, 2H), 7.70–7.74 (m, 2H), 7.83–7.85 (m, 2H)
J5	1.34–1.48 (m, 6H), 1.64–1.79 (m, 4H), 2.99 (d, 3H), 3.11 (d, 3H), 3.66–3.71 (t, 2H), 3.93–3.98 (t, 2H), 6.87–6.93 (t, 2H), 7.06–7.15 (m, 2H), 7.69–7.72 (m, 2H), 7.82–7.85 (m, 2H)
J6	1.35–1.45 (m, 8H), 1.65–1.78 (m, 4H), 3.00 (s, 3H), 3.12 (s, 3H), 3.65–3.70 (t, 2H), 3.93–3.97 (t, 2H), 6.87–6.92 (t, 2H), 7.06–7.15 (m, 2H), 7.69–7.74 (m, 2H), 7.81–7.85 (m, 2H)
J7	1.21–1.41 (m, 10H), 1.65–1.78 (m, 4H), 3.00 (s, 3H), 3.12 (s, 3H), 3.65–3.70 (t, 2H), 3.93–3.98 (t, 2H), 6.87–6.93 (t, 2H), 7.06–7.16 (m, 2H), 7.69–7.74 (m, 2H), 7.82–7.86 (m, 2H)
J8	1.65–1.76 (m, 4H), 3.00 (s, 3H), 3.12 (s, 3H), 3.65–3.70 (t, 2H), 3.93–3.98 (t, 2H), 6.88–6.93 (t, 2H), 7.06–7.16 (m, 2H), 7.69–7.72 (m, 2H), 7.81–7.86 (m, 2H)
K1	2.13–2.21 (m, 2H), 3.00 (s, 3H), 3.08 (s, 3H), 3.87–3.92 (t, 2H), 3.96–4.03 (t, 2H), 6.59–6.71 (m, 3H), 7.16–7.27 (m, 1H), 7.70–7.73 (m, 2H), 7.83–7.85 (m, 2H)
K2	1.81–1.89 (m, 4H), 3.00 (s, 3H), 3.09 (s, 3H), 3.74–3.78 (t, 2H), 3.95–3.99 (t, 2H), 6.64–6.73 (m, 3H), 7.18–7.26 (m, 1H), 7.70–7.73 (m, 2H), 7.83–7.86 (m, 2H)
K3	1.46–1.56 (m, 2H), 1.70–1.86 (m, 4H), 3.00 (s, 3H), 3.09 (s, 3H), 3.69–3.74 (t, 2H), 3.90–3.95 (t, 2H), 6.64–6.73 (m, 3H), 7.18–7.27 (m, 1H), 7.68–7.73 (m, 2H), 7.82–7.86 (m, 2H)
K4	1.37–1.53 (m, 4H), 1.66–1.78 (m, 4H), 3.00 (s, 3H), 3.09 (s, 3H), 3.67–3.72 (t, 2H), 3.90–3.94 (t, 2H), 6.64–6.73 (m, 3H), 7.19–7.26 (m, 1H), 7.69–7.72 (m, 2H), 7.83–7.86 (m, 2H)
K5	1.40–1.43 (m, 6H), 1.64–1.77 (m, 4H), 3.00 (s, 3H), 3.09 (s, 3H), 3.66–3.71 (t, 2H), 3.89–3.93 (t, 2H), 6.65–6.74 (m, 3H), 7.19–7.27 (m, 1H), 7.69–7.72 (m, 2H), 7.82–7.85 (m, 2H)
K6	1.35–1.44 (m, 8H), 1.67–1.77 (m, 4H), 3.01 (s, 3H), 3.09 (s, 3H), 3.66–3.71 (t, 2H), 3.89–3.93 (t, 2H), 6.65–6.74 (m, 3H), 7.19–7.27 (m, 1H), 7.69–7.73 (m, 2H), 7.81–7.86 (m, 2H)
K7	1.32–1.44 (m, 10H), 1.65–1.77 (m, 4H), 3.01 (s, 3H), 3.09 (s, 3H), 3.65–3.70 (t, 2H), 3.89–3.93 (t, 2H), 6.65–6.74 (m, 3H), 7.19–7.26 (m, 1H), 7.69–7.73 (m, 2H), 7.81–7.86 (m, 2H)
K8	1.29–1.42 (m, 12H), 1.65–1.77 (m, 4H), 3.01 (s, 3H), 3.09 (s, 3H), 3.65–3.70 (t, 2H), 3.89–3.94 (t, 2H), 6.65–6.75 (m, 3H), 7.19–7.267 (m, 1H), 7.69–7.71 (m, 2H), 7.82–7.85 (m, 2H)

Table 3
Inhibition of AChE activities and ratios to the standard compounds.

Compd	IC ₅₀ (95% confidence intervals) (μM)	Ratio to PDM ^a
PDM	2961(2344–3742)	1
J1	90.5(69.8–117.4)	31.16
J2	546(345–864)	5.42
J3	1759(637–4859)	0.54
J4	>2500	—
J5	>2500	—
J6	>2500	—
J7	>2500	—
J8	>2500	—
K1	7.33(5.87–9.14)	404.29
K2	197(176–222)	15.02
K3	206(156–272)	14.37
K4	126(98–163)	23.49
K5	157(111–220)	18.85
K6	240(213–270)	12.33
K7	185(60–576)	16.00
K8	461(190–1116)	6.42

^a Ratio to PM was defined as: IC₅₀(PDM)/IC₅₀(compounds **J** or **K**).

As the distance between the esteratic and anionic sites was 0.5 nm in the AChE molecule, carbamate insecticides would be most efficient if the distance between the two groups to be bound to the two sites of the enzyme was also 0.5 nm [7,12,13]. Compounds **J1**, **J2** and **K1–K8** showed higher activity than that of PDM, in which the substituents on the phenyl ring were longer than 0.5 nm to extent halfway into the gorge. It suggested the phthalimide groups of these compounds interacted with another residues lining the gorge to promote the affinity of the molecules.

The variations of number of methylene units of linkage exhibited obvious effect on the AChE inhibitory activity. When *n* was 3, the compounds had the highest AChE inhibitory activity. The inhibitory activity dramatically decreased, while the methylene units of the linker was changed from three to four and four to five, which indicated that some unfavorable interaction strongly affected their binding to the AChE gorge. Different activity of the compounds might be caused by the different binding sites of phthalimide group into AChE, or adding of the chain length of methylene units, which increased both hydrophobicity and steric hindrance of the molecules, reduced the inhibitory activity on isolated AChE.

The most potent AChE inhibitory activity to isolated enzyme was observed for the compound **K1** with the IC₅₀ values of 7.33 μM, which had the best activity (404-fold higher than PDM). Compound **J1** exhibited 31-fold AChE inhibitory activity higher than that of PDM, also. These results demonstrated that phthalimide propoxy of compound **K1** interacted with the residues tightly. The structure of compound **K1** was in accordance with the enzyme gorge in the best way while phthalimide propoxy was at *meta*-position of phenyl group.

In conclusion, the results demonstrated the feasibility of dual-site inhibitors on isolated AChE from head of housefly of suscepti-

ble strains. The compounds **K1–K8** demonstrated higher inhibitory activity for AChE than for PDM while the substituent was in the *meta*-position. Compound **K1** showed the highest activity, in which a suitable large substituent of phenyl *N,N*-methylcarbamate binding the residues lining the gorge well increased the AChE inhibitory ability enormously. Further research about in vivo bioassay was underway.

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