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Acetylcholinesterase/Butyrylcholinesterase inhibition activity of some new carbacylamidophosphate deriviatives

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Abstract

Eight newly synthesized carbacylamidophosphates with the general formula $RC(O)NHP(O)Cl_2$ with R═pCl—C_6H_4 **1a**, pBr—C_6H_4 **2a**, C_6H_5 **3a**, and pMe—C_6H_4 **4a** and $RC(O)NHP(O)(NC_4H_8O)_2$ R═pCl—C_6H_4 **1b**, pBr—C_6H_4 **2b**, C_6H_5 **3b**, pMe—C_6H_4 **4b**, were selected to compare the inhibition kinetic parameters, IC_{50}, K_i, k_p and K_D, on human erythrocyte acetylcholinesterase (hAChE) and bovine serum butyrylcholinesterase (BuChE), Also, the *in vivo* inhibition potency of compound **2a**, **2b** and **3a**, were studied. The data demonstrates that compound **2a** and compound **2b** are the potent sensitive as AChE and BuChE inhibitors respectively, and the inhibition of hAChE is about 10-fold greater than that of BuChE.

Keywords: *Carbacylamidophosphates, acetylcholinesterase, butyrylcholinesterase IC_{50}, K_i, k_p, K_D*

Introduction

Mammals have two main cholinesterases: acetylcholinesterase (AChE, E.C. 3.1.1.7) and butyrylcholinesterase (BuChE, E.C. 3.1.1.8). They are inhibited by two main groups of pesticides, the organophosphates and the carbamates, which combine covalently with specific amino-acid residues to inactivate the enzyme. These distinct enzymes possess differences in their tissue distribution, kinetic properties, specificity for synthetic and natural substrates and selective inhibitors [1–4] although they are evolutionarily related. In humans, AChE is more abundant in the central nervous system, end plate of skeletal muscle and erythrocyte membranes while BuChE is more abundant in serum [5].

BuChE, also called nonspecific cholinesterase or pseudocholinesterase, is able to act on hydrophilic and hydrophobic choline esters [6]. At this moment, the exact physiological function of BuChE is not yet clear, but it is well known that this enzyme hydrolyses a variety of xenobiotics such as aspirin, succinylcholine, heroin and cocaine [7]. Recently, it was suggested that BuChE was found located with senile plaques in the central nervous system, and plays a role in the progressive H-amyloid aggregation and in senile plaques maturation [8].

AChE is an important regulatory enzyme that controls the transmission of nerve impulses across cholinergic synapses by hydrolyzing the excitatory transmitter acetylcholine (ACh) [9,10]. Of toxicological and pharmacological significance, AChE is a target for various cholinergic toxins, such as natural snake venom and plant glycoalkaloids, and also a target for therapeutically active compounds, including anti-Alzheimer's disease drugs. However, there is growing evidence that cholinesterase, probably through their 'noncholinergic' functions, could participate in the pathological processes in Alzheimer's disease such as the H-amyloid formation or deposition [11]. In this way, ChE inhibitors may modulate the processing

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of h-amyloid protein, thus reducing the deposition of h-amyloid itself [12]. The enzyme reaction catalyzed by acetylcholinesterase is one of the most efficient reactions known. This efficiency could be due to the three-dimensional structure of the enzyme. The long and narrow active site gorge is about 20 Å deep and includes two sites of ligand interaction: an acylation site at the base of the gorge with the catalytic triad and a peripheral site at its mouth [13]. Some ligands can bind specifically to the acylation or to the peripheral site, and ternary complexes with different ligands bound to each site can be formed.

In BuChE and AChE, the hydrolysis is carried out by a "catalytic triad" of Ser, His and Glu in the active center [14]. Therefore, there are some differences in the catalytic course; for example, BuChE possesses a larger volume in its active site and can be a key factor in determining substrate preference and inhibitor affinity [15].

In our previous studies we discussed the synthesis and inhibition potency of some new phosphoramidate compounds and determined *in vitro* IC_{50} of them [16,17]. In this work we considered the inhibition potency of 8 new synthesized carbacylamidophosphates [18,19] with general formula $RC(O)NHP(O)(R')_2$ with R=Cl—C_6H_4, Br—C_6H_4, C_6H_5 and Me—C_6H_4, and R′=Cl and morpholine; on human erythrocyte acetylcholinesterase and bovine erythrocyte butyrylcholinesterase activity (Scheme 1). *In vitro* activity of hAChE (pure and crude) in reaction with two class of carbacylamidophosphete (**1a**–**4a**) and (**1b**–**4b**) was measured, and its kinetic parameters (inhibitory potency (IC_{50}), biomolecular rate constants (k_i), dissosiation constant (K_D) and phosphorylation constant (k_p)) were determined. Furthermore, hydrophobicity of these compounds was characterized by octanol/water partition coefficient (log P) and theoretical method. Finally, *in vivo* 50% inhibitory activity of three compounds; **2a**, **2b** and **3b** on AChE and BuChE activity were determined and acute toxicity (LD_{50}) of these compounds also was studied.

Experimental

Chemicals

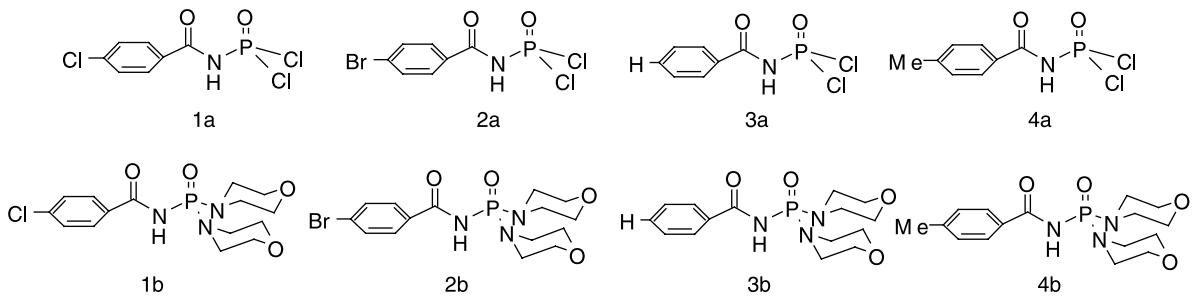
All reactions in the synthesis of compounds from 1 to 8 based on published methods [17,18], were carried out. Purified human erythrocytes AChE (3.1.1.7; 50 units/785 μL), purified horse plasma BuChE (lyophilized) from Sigma-Aldrich (UK), butyrylthiocholine (BTCh) iodide, acetylthiocholine (ATCh) iodide, and 5,4′-dithio bis (2-nitrobenzoic acid) (DTNB) from Fluka (Tehran) were used. All remaining chemicals and solvents were purchased from Merck (Tehran).

In vitro *experiments*

AChE activity assay. The activity of AChE was determined by a modified Ellman's method [27] as the level of ASCh hydrolysis by monitoring thiocholine liberated and reacted with DTNB. Reactions were carried out at 37°C in 70 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH = 7.4, 920 μL) containing the enzyme (diluted 100 times in phosphate buffer, pH = 7.4), DTNB (0.1 mM final concentration, 50 μL), and ASCh (0.135 mM final concentration, 15 μL). The absorbance change at 37° C was monitored with the spectrophotometer at 412 nm for 3 min and three replicates were run in each experiment. In the absence of inhibitors, the absorbance change was directly proportional to the enzyme level.

BuChE activity assay. The activity of BuChE was determined the same as AChE activity by measuring thiocholine reacted with DTNB after hydrolysis of BuSCh. The lyophilized BuChE was diluted with 100 mM phosphate buffer (pH = 8) for using in activity assay.

AChE and BuChE inhibition studies. The reaction mixtures for determination of IC_{50} values of human erythrocyte acetylcholinesterase (hAChE), consisted DTNB solution, 5 μL; inhibitor, x μl ($5 < x < 15$); acetylthiocholine iodide (ASCh) solution, 10 μL; and



Scheme 1.

phosphate buffer (pH = 7.4) for a final volume of 100 μL. The final concentrations of DINB and ATCh were 10^{-4} and 2.7×10^{-5} M. The enzyme concentration in the assay was 1.004×10^{-10} M. Also, the reaction mixtures for IC_{50} values determination for BuChE were the same as hAChE assay. The candidate inhibitors were incubated with the enzyme (hAChE or BuChE), inhibitors, and DTNB for 20 min at 37°C prior to addition of ASCh or BuSCh for residual activity assay. The samples were placed in 96 ELISA well dishes (Nunc, Denmark) and the results were obtained by placing 96-well dishes into ELISA plate reader (Stat-Fax 303 plus, Awareness Technology Inc, palm city, FL) at 412 nm and the data were collected every 40 s for 3 min.

Measurement of hydrophobicity. The experimental hydrophobic parameter of compounds was determined by measurement of octanol–water partition coefficient by shake-flask technique [28–32]. The aqueous phase stock solution were shaken with excess of octanol to presaturate them and were allowed to stand overnight before use. The octanol stock solutions were also presaturated with water, allowed to settle overnight and stored at 25°C as was done with the aqueous phase. Then three concentrations (0.001, 0.01, 0.05 M) of solutes were prepared in octanol and then 2.5 mL of these solutions was added to 500 mL water and shaken well and was allowed to stand 30 min, then, two phases were separated and GC instrument was used to determine the concentration of compounds in octanol and water phase by internal standard method.

Calculated log P, hydrophobicity extent, of these eight synthesized molecules are performed using software log P, *ChemDraw Ultra, 8.0.3, 2003*.

In vivo *experiments*

Animals. NMRI albino mice weighing between 20–25 g were obtained from Pharmaceutical Sciences Research Center (PSRC) of Tehran University of Medical Sciences (TUMS) and experiments were conducted in accordance with University protocols. The animals were all individually housed in plastic cages in an air-conditioned room with controlled temperature (20–22°C) and automatic lighting and free access to standard laboratory diet and water. Animals were maintained under this condition for 10 days prior to experimentation. They were randomly divided into three groups consisting four mice in each. Regarding the inhibitory concentration (IC_{50}) of the reference compounds such as paraoxon (4.7 ± 1.2 μM), the equimolar dose and two upper and lower doses of the compounds **2a**, **2b** and **3a**, in a 1.5 order, were used in mice. Therefore, animals from group one to three received doses of 7.05, 4.7 and 3.13 μM equal to 1.72, 1.15, and 0.76 mg/kg of the compounds **2a**, **2b** and **3a** as the most potent inhibitors, by gavage.

AChE activity in erythrocytes. The rate of hydrolysis of ATCh iodide in the suspension of erythrocytes (pH 7.6) in the presence of benzetonium chloride was determined by measuring the maximum absorbance at 440 nm. In this test, the reaction of thiocholine iodide with DTNB gives a yellow 5-thio-2-nitrobenzoate anion. The enzyme activity was expressed as KU/L [18].

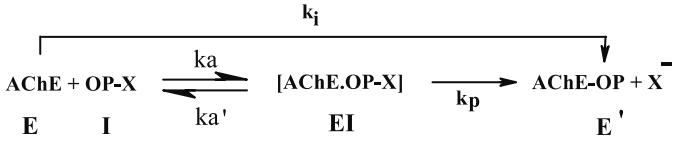
BuChE activity in plasma. Ten microliters of plasma sample were added to each duplicate tube, containing 3 mL of 25 nM DTNB in 75 mM phosphate buffer. Then 10 μL of 3 mM butyrylthiocholine iodide were added to the sample tube, and change in the absorbance was measured at 412 nm [18].

Determination of Toxicity (LD_{50}). In order to determine the acute toxicity (LD_{50}) of the compounds, various doses (100, 300, 600, 1000, 1500, and 2000 mg/kg/day) were gavaged to NMRI albino mice in separate groups of 4 in each. The animals were observed for 48 h and any mortality was recorded at the end of this period. The LD_{50} was determined by regression probit using Stats Direct [29].

Results

In vitro *studies*

Derivation of the kinetic values was based on the work of Kitz and Wilson (1962) and Segel (1975) [20,21].



$$ka[I][E] = ka'[EI] + kp[EI]$$

$$Et = E + EI + E' \quad \text{if} \quad \varepsilon = E + EI \quad \text{and} \quad km = \frac{ka' + kp}{ka}$$

$$[EI] = \frac{\varepsilon}{\frac{km}{[I]} + 1}$$

$$\frac{-d(\varepsilon)}{dt} = kp[EI] \quad \text{if} \quad k_{app} = \frac{kp}{\frac{km}{[I]} + 1}$$

$$\ln\left(\frac{\varepsilon}{\varepsilon_0}\right) = -k_{app}(t)$$

K_D is the dissociation constant, k_p is the rate constant of AChE phosphorylation, and K_i is the bimolecular inhibition constant. The plot of v_i/v_o (v_i and v_o are the activities of the enzyme in the presence and absence of inhibitor, respectively) against log [I], where [I] is the inhibitor concentration, gave the IC_{50} values of isolated compounds. Least squares linear regression (ln) of the fraction of remaining AChE activity (vi/v0) versus time (min) resulted in a line at each inhibitor concentration with slope $= -k_{app}$ (the apparent rate of AChE inhibitor phosphorylation). A double reciprocal plot of the inhibitor concentration versus k_{app} resulted in a line with slope $= 1/K_i$, y-intercept $= 1/k_P$ and x-intercept $= -1/K_D$.

The *in vitro* inhibitions of hAChE for compounds **1a**–**4a** and **1b**–**4b** are presented in Figure 1. Also, the Figure 2 represents the *in vitro* inhibition of BuChE for compounds **1a**–**4a** and **1b**–**4b** and the IC_{50} values are included in Tables I and II. The data demonstrate that compound **2a** from series **1a**–**4a** and compound **2b** from series of **1b**–**4b** are most potent as AChE and BuChE inhibitors. By comparing the IC_{50} of these compounds, it is clear that the inhibition potency of these compounds in hAChE is nearly 10-fold greater than BuChE inhibition. Furthermore, compounds **1a**–**4a** have more inhibitory effect on both hAChE and BuChE than compounds **1b**–**4b**. The representative set of data for determining $-k_{app}$ at different concentrations of inhibitors **1a**–**4a** and **1b**–**4b** for human erythrocyte AChE are presented in Figures 3 and 4. Also, Figures 5 and 6 represent a set of data for determining $-k_{app}$ at diffrent concentrations of inhibitors **1a**–**4a** and **1b**–**4b** for horse plasma BuChE. Least squares linear regression of the data at each inhibitor concentration resulted in a line having slope $= -k_{app}$ with regression coefficients from 0.98 to 0.99. As the concentration of these compounds increased, the slope increased, resulting in larger K_{app} values. A double reciprocal plot of the inhibitors concentration versus the K_{app} values from Figures 3–6 resulted in a line with slope $= 1/K_i$, y-intercept $= 1/kp$ and x-intercept $= -1/K_D$ and regression coefficients between 0.98 to 0.99 (Figures 7 and 8). The K_i, k_p and K_D values for all compounds are calculated for hAChE and BuChE and are summarized in Tables I and II. These data suggest that the inhibition of AChE and BuChE follows a pseudo-first order Michaelis-Menten process. Comparing the kinetics (IC_{50}, K_i, kp and K_D) of AChE and BuChE inactivation by selected compounds resulted in no significant difference for the measured variables except for compounds **2a** and **2b**,

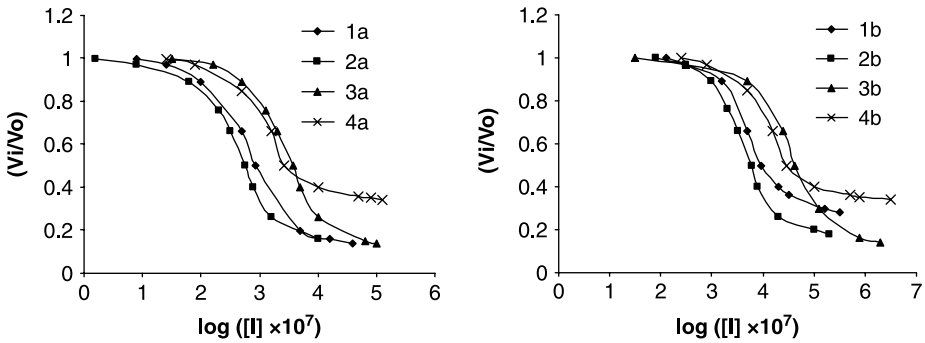


Figure 1. The plot of v_i/v_0 aganst log ([I] × 10^7) for inhibitor **1a**–**4a** and **1b**–**4b**: v_i and v_0 are the activity of human erythrocyte AChE in the presence and absence of inhibitor, respectively and [I] is inhibitor concentration (M).

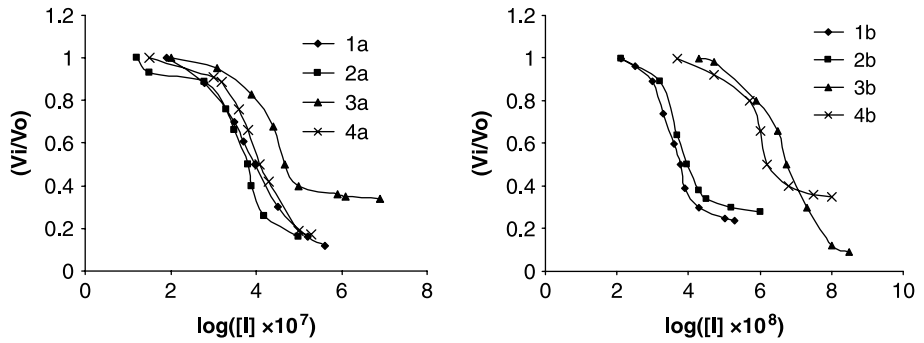


Figure 2. The plot of v_i/v_0 aganst log ([I] × 10^7) for inhibitors **1a**–**4a** and log ([I] × 10^8) for inhibitors **1b**–**4b**: v_i and v_0 are the activity of horse plasma BuChE in the presence and absence of inhibitor, respectively and [I] is inhibitor concentration (M).

Table I. Kinetics of human erythrocyte AChE inactivation by compounds (**1a**–**4a**) and (**1b**–**4b**).

Compound	IC50 (μM)	k_i ($\mu M^{-1} min^{-1}$)	k_p (min^{-1})	K_D (μM)
(p—Cl—C_6H_4)C(O)NHP(O)$(Cl)_2$,**1a**	87 ± 3.4⋆	0.0598 ± 0.0	12.33 ± 1.7	206 ± 6.4
(p—Br—C_6H_4)C(O)NHP(O)$(Cl)_2$,**2a**	57 ± 2.7	0.0672 ± 0.0	15.06 ± 1.3	230 ± 7.1
(C_6H_5)C(O)NHP(O)$(Cl)_2$,**3a**	385 ± 8.9	0.00956 ± 0.0	11.13 ± 0.9	1100 ± 12.3
(p—Me—C_6H_4)C(O)NHP(O)$(Cl)_2$,**4a**	260 ± 7.3	0.0168 ± 0.0	9.31 ± 0.5	554 ± 8.6
(p—Cl—C_6H_4)C(O)NHP(O)$(NC_4H_8O)_2$,**1b**	932 ± 15.2	0.00692 ± 0.0	1.65 ± 0.1	239 ± 7.5
(p—Br—C_6H_4)C(O)NHP(O)$(NC_4H_8O)_2$,**2b**	620 ± 10.5	0.00911 ± 0.0	3.48 ± 0.2	382 ± 8.4
(C_6H_5)C(O)NHP(O)$(NC_4H_8O)_2$,**3b**	4150 ± 64.8	0.000769 ± 0.0	2.27 ± 0.3	2954 ± 10.2
(p—Me—C_6H_4)C(O)NHP(O)$(NC_4H_8O)_2$,**4b**	2890 ± 42.5	0.000956 ± 0.0	4.07 ± 0.2	4260 ± 15.3

⋆Values represent the means ± SD of three individual parameters determination.

Table II. Kinetics of BuChE inactivation by compounds (**1a**–**4a**) and (**1b**–**4b**).

Compound	IC50 (μM)	Ki ($\mu M^{-1} min^{-1}$)	Kp (min^{-1})	KD (μM)
(p—Cl—C_6H_4)C(O)NHP(O)$(Cl)_2$,**1a**	920 ± 13.5⋆	0.00418 ± 0.0	18.11 ± 2.1	43000 ± 124
(p—Br—C_6H_4)C(O)NHP(O)$(Cl)_2$,**2a**	650 ± 11.2	0.00734 ± 0.0	15.13 ± 1.7	2100 ± 14.2
(C_6H_5)C(O)NHP(O)$(Cl)_2$,**3a**	4800 ± 75.3	0.000755 ± 0.0	14.02 ± 1.4	18000 ± 75.6
(p—Me—C_6H_4)C(O)NHP(O)$(Cl)_2$,**4a**	1200 ± 25.6	0.00192 ± 0.0	17.12 ± 1.8	8900 ± 213
(p—Cl—C_6H_4)C(O)NHP(O)$(NC_4H_8O)_2$,**1b**	9980 ± 130.7	0.000368 ± 0.0	1.86 ± 0.1	5063 ± 17.5
(p—Br—C_6H_4)C(O)NHP(O)$(NC_4H_8O)_2$,**2b**	5240 ± 80.2	0.000453 ± 0.0	1.82 ± 0.1	4370 ± 16.2
(C_6H_5)C(O)NHP(O)$(NC_4H_8O)_2$,**3b**	55620 ± 986	0.0000684 ± 0.0	5.26 ± 0.4	76804 ± 241
(p—Me—C_6H_4)C(O)NHP(O)$(NC_4H_8O)_2$,**4b**	16720 ± 274	0.000140 ± 0.0	4.70 ± 0.6	33557 ± 163

⋆Values represent the means ± SD of three individual parameters determination.

which appeared to be significantly more potent to hAChE and BuChE with a lower IC_{50} in comparison with the other compounds.

As shown in Tables II and III, the IC_{50} value of compound **2a** and **2b** is nearly 2 to 6-fold lower than the other structurally related compounds.

Calculated log P values, the extent of hydrophobicity, of selected inhibitors are presented in Tables III and IV and the experimental data, characterized by octanol/water partition coefficient (log P). The results show that, compound **2a** is the most hydrophobic molecule relative to the other compound.

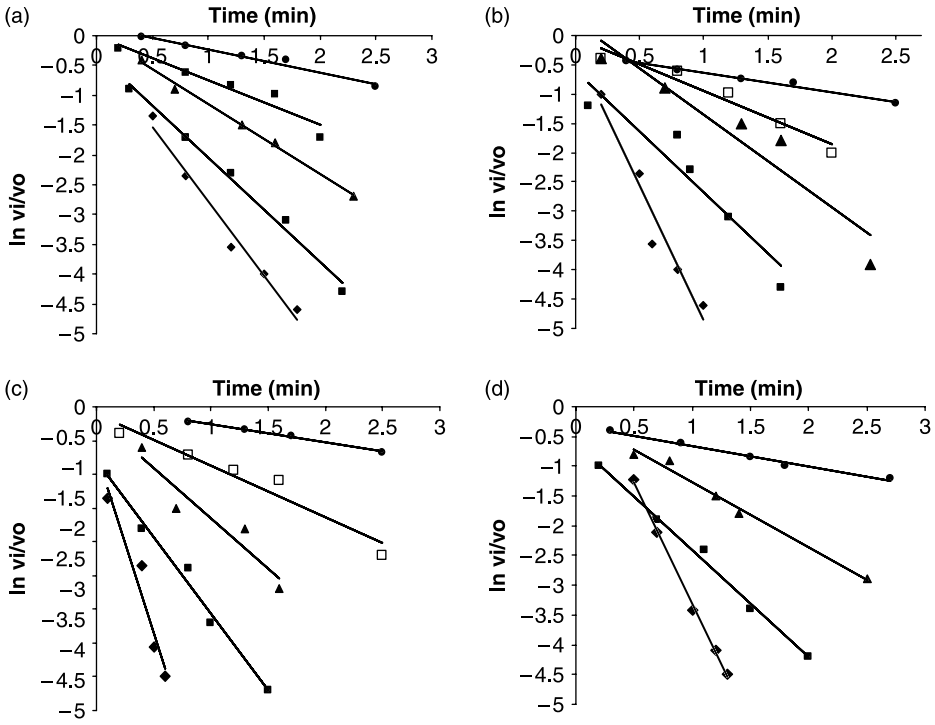


Figure 3. A representative set of data for determining −kapp at diffrent concentrations of inhibitors **1a**–**4a** for human erythrocyte AChE. The time of incubation was variable for each inhibitor concentration and the slope of the lines (− kapp) increased with increasing inhibitor concentration. Inhibitor concentrations (μM) were, **1a**: 6, 9, 15, 30, 60; **2a**: 5, 10, 20, 30, 100; **3a**: 20, 60, 80, 100, 200; **4a**: 20, 50, 100, 200.

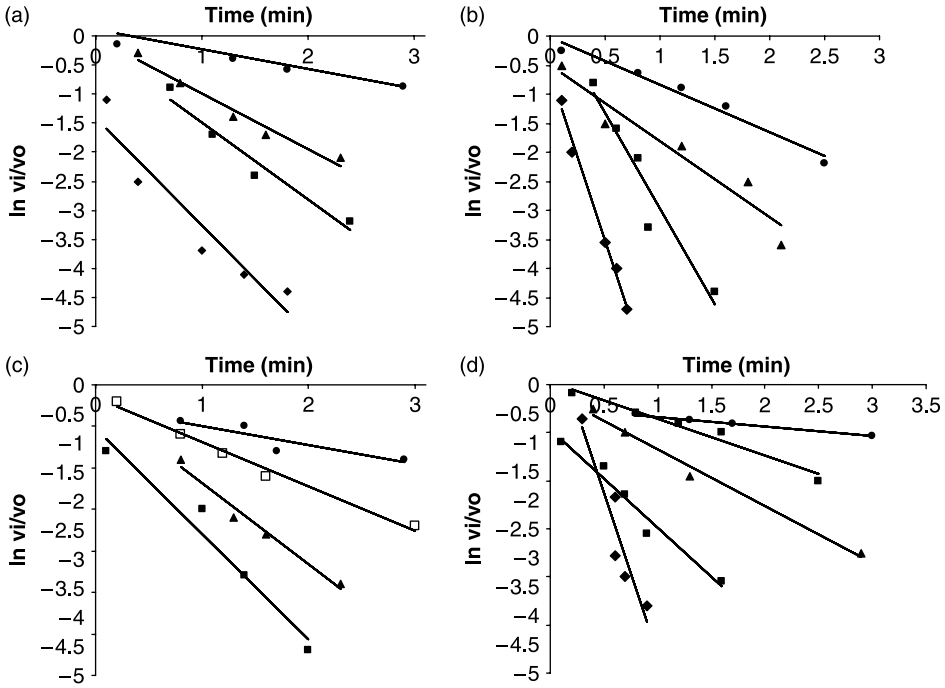


Figure 4. A representative set of data for determining −kapp at diffrent concentrations of inhibitors **1b**–**4b** for human erythrocyte AChE. The time of incubation was variable for each inhibitor concentration and the slope of the lines (−kapp) increased with increasing inhibitor concentration. Inhibitor concentrations (μM) were, **1b**: 40, 80, 100, 150; **2b**: 70, 100, 150, 250; **3b**: 300, 1000, 2000, 3000; **4b**: 200, 500, 1000, 1500, 3000.

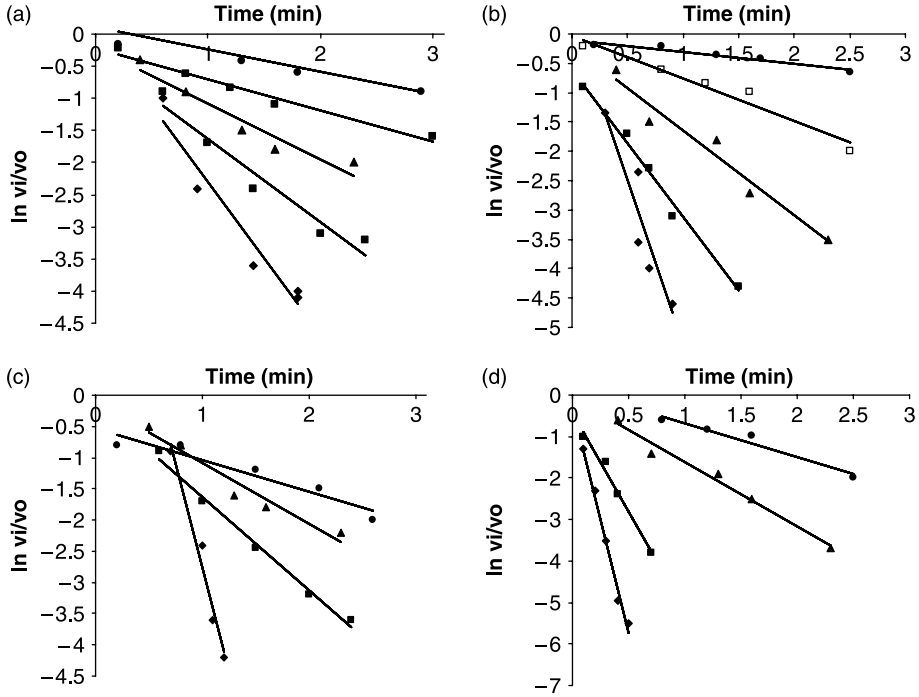


Figure 5. A representative set of data for determining −kapp at diffrent concentrations of inhibitors **1a**–**4a** for horse plasma BuChE. The time of incubation was variable for each inhibitor concentration and the slope of the lines (−kapp) increased with increasing inhibitor concentration. Inhibitor concentrations (μM) were, **1a**: 80, 100, 180, 200, 500; **2a**: 30, 80, 150, 250, 500; **3a**: 600, 1500, 2500, 5000; **4a**: 300, 600, 1000, 1500.

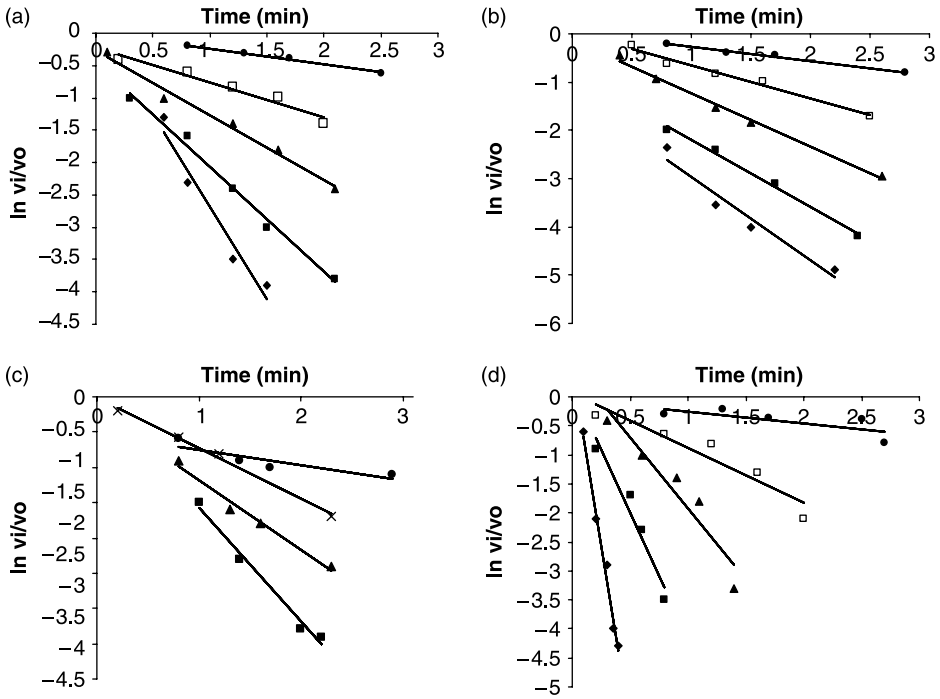


Figure 6. A representative set of data for determining −kapp at diffrent concentrations of inhibitors **1b**–**4b** for horse plasma BuChE. The time of incubation was variable for each inhibitor concentration and the slope of the lines (−kapp) increased with increasing inhibitor concentration. Inhibitor concentrations (μM) were, **1b**: 600, 900, 1500, 2500, 5000; **2b**: 600, 1000, 1500, 1700, 2500; **3b**: 3000, 10000, 20000, 35000; **4b**: 1500, 5000, 10000, 20000, 50000.

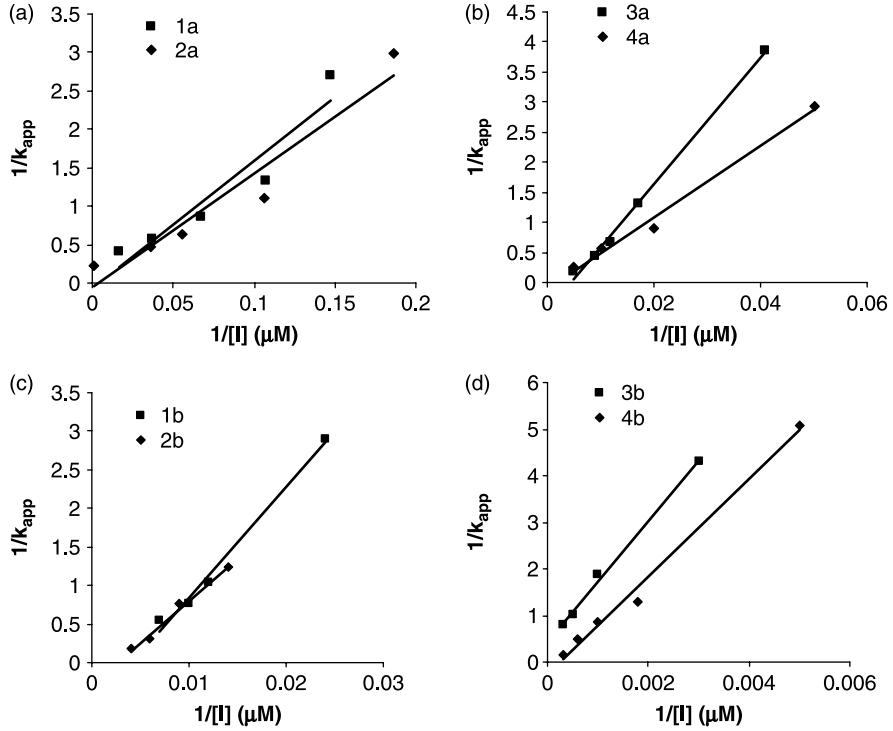


Figure 7. A representative double reciprocal plot of different concentrations of inhibitors (**1a**–**4a** and **1b**–**4b**) versus K_{app} from Figures 3 and 4. Linear regression of representing kapp at different concentrations of inhibitor concentrations for each inhibitor resulted a line with slope = $1/K_i$, y-intercept = 1/kp, and x-intercept = $-1/K_D = -K_A$.

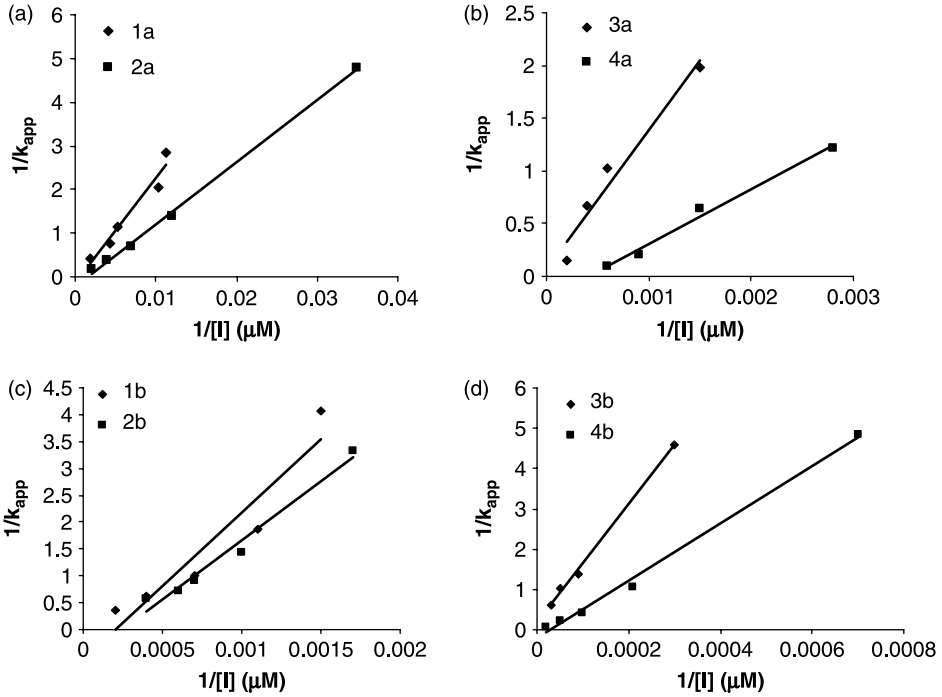


Figure 8. A representative double reciprocal plot of different concentrations of inhibitors (**1a**–**4a** and **1b**–**4b**) versus K_{app} from Figures 5 and 6. Linear regression of representing kapp at different concentrations of inhibitor concentrations for each inhibitor resulted a line with slope = $1/K_i$, y-intercept = 1/kp, and x-intercept = $-1/K_D = -K_A$.

In vivo *studies*

The acute toxicity test (LD_{50}) demonstrated that compounds (**2a**, **2b** and **3a**) are lethal up to a dose of <1000 mg/kg after oral administration (Table IV).

Treatment with compounds **2a**, **2b** and **3a** in doses of <100 (mg/kg/day) inhibited both AChE activity in erythrocytes and BuChE activity in plasma (Table V), where **2a** had greater potent inhibition activity ($IC_{50} = 130$ μM) than the other compounds (**2b** and **3a**). As shown in Table IV, AChE was more sensitive to these compounds in comparison to BuChE.

Chemistry

Some spectroscopic data for candidate compounds is presented in Table V. The ^{31}P chemical shifts of compounds (**1a**–**4a**) with Cl subtituents are in up fields relative to their analogous compounds (**1b**–**4b**) with morpholine group. The **1a**–**4a** analogues exhibit a single $^{31}P\{^1H\}$ NMR resonance between 11.29 to 11.52 ppm, however these value for **1b**–**4b** is in the range 10.81 to 10.87 ppm.

Some absorption bands in the IR spectra of the selected compounds, together with their assignments are given in Table I. The very strong absorption band observed around 1191 to 1232 cm^{-1} belongs to the stretching vibrations of P═O bond in **1a**–**4a**. The stretching vibrations of υ(P═O) in **1b**–**4b** appear in the range 1191–1195 cm^{-1}. IR spectra of the **1a**–**4a** compounds exhibit the characteristic band of carbonyl group (C═O), which appears in the region 1684–1680 cm^{-1}. This band is shifted to lower frequencies in the IR spectra of **1b**–**4b** compounds, cm^{-1}(1662–1666).

Table III. log P of selected inhibitors.

Compound	log P (Theoretical)	*log P (Experimental)
1a	3.25	5.21 ± 0.6
2a	2.97	6.55 ± 0.7
3a	2.45	4.23 ± 0.6
4a	2.99	5.68 ± 0.7
1b	1.54	3.11 ± 0.4
2b	1.26	3.54 ± 0.5
3b	0.74	1.32 ± 0.1
4b	1.21	2.99 ± 0.2

*Values represent the means ± SD of three individual parameters determination.

Discussion

In vitro *study*

Two classes of isostructure phosphoramidate compounds **1a**–**4a** and **1b**–**4b**, with —(CO)NHP(O)— group were selected which have likely β-diketone skeleton. The inhibitory potency of candidate compounds was compared with respect to its electronic effects and hydrophobicity.

Electronic effects. As shown in Tables II and III, the IC_{50} and k_i values indicate that the replacement of Cl from

Table IV. *In vivo* experiments‡.

Compound	LD_{50} (mg/kg)	IC_{50}⋆ (μM)	IC_{50}† (μM)
(p—Br—C_6H_4)C(O)NHP(O)$(Cl)_2$,**2a**	150 ± 10.5⋆	130 ± 9.2	1600 ± 18.3
(C_6H_5)C(O)NHP(O)$(Cl)_2$,**3a**	600 ± 15.3	820 ± 14.1	9900 ± 22.6
(p—Br—C_6H_4)C(O)NHP(O)$(NC_4H_8O)_2$,**2b**	240 ± 13.8	970 ± 13.7	12000 ± 142

⋆AChE; †BuChE; ‡Values represent the means ± SD of three individual parameter determination.

Table V. Some spectroscopic data for compounds (**1a**–**4a**) and (**1b**–**4b**).

Compound	**1a**	**2a**	**3a**	**4a**	**1b**	**2b**	**3b**	**4b**
M_W(g/mol)	272.45	316.6	238.01	252.00	373.5	418.00	339.35	353.35
$^{31}\delta$P(ppm)	11.41	11.47	11.52	11.29	10.87	10.81	10.83	10.81
$^2J_{PNH(amide)}$ (Hz)	12.03	12.01	12.00	11.9	5.5	5.2	5.3	5.9
$\delta NH_{(amide)}$ (ppm)	9.91	9.89	9.77	9.88	8.05	8.82	8.41	8.18
ν P═O(cm^{-1})	1226	1232	1221	1230	1193	1191	1194	1195
υ(C═O) (cm^{-1})	1683	1684	1682	1680	1666	1667	1661	1662

the P—Cl moiety of compound **1a**–**4a** by morpholine group decreased the affinity toward the active site of human erythrocyte AChE. Reactivity of phosphoramidate compounds toward nucleophiles is believed to increase with increasing of the electrophilicity of phosphorus atom. So the electronic properties of the compounds should be important in determining inhibitory potency. The charge of phosphorous atom in carbacylamidophosphates compounds can be inferred from the spectroscopic data. ^{31}P chemical shift is a parameter that can indicate electron density around the phosphorus nuclei. The ^{31}P NMR spectra for **1b**–**4b** exhibited a ^{31}P NMR signal which is shielded relative to the **1a**–**4a** that indicates a greater electron density around phosphorus atom in these compounds.

The ^{1}H NMR spectra of these compounds presented noticeable information about the coupling of the amidic proton with the phosphorus atom ($^2J_{PNH}$). $^2J_{PNH}$ in the intermediate compounds, **1a**–**4a** appear in rang of 11.9 to 12.03 Hz. However, replacement of chlorine substituents by morpholine groups decreased the same $^2J_{PNH}$ values for the amide nitrogen.

These $^2J_{PNH}$ values demonstrate, in compounds **1a**–**4a** that a strong interaction between lone pairs of amidic nitrogen and phosphorus atom, however substitution of chlorine atom by morpholine in **1b**–**4b** decreased this interaction and thereby decreased the $^2J_{PNH}$ values. This information indicates high electron density in **1b**–**4b** that cause decreasing inhibitory potency of these compounds.

The next important factor is lability of leaving group. Inhibition of AChE by phosphoramidate compounds is result of the ejection of leaving group and the active site of AChE phosphorylates by phosphoryl residue [22–24]. It was known that chlorine atom is the labile leaving group. This matter is confirmed by thermochemical bond energies in phosphorus compounds [25]. Hence, inhibition of hAChE by **1a**–**4a** probably was resulted from lability of P—Cl bond. The IC_{50} values indicate that the replacement of Chlorin atom of compounds **1a**–**4a** by nitrogen atom that has high thermochemical bond energies, in **1b**–**4b** decreased the affinity toward the active site of human erythrocyte AChE. Therefore different inhibitory potency of these two classes of inhibitors, in addition of charge on phosphorous atom can be relevant to the lability of leaving group of synthesized molecules.

The IC_{50} values of the isostructure phosphoramidates (**1a**–**4a**) indicates that the increase in the inhibitory effect on hAChE is **2a** > **1a** > **4a** > **3a**. The ^{31}P NMR spectra for these compounds showed small difference between ^{31}P chemical shifts. So, electronegativity of benzene ring substituents probably, (Me, H, Br and Cl) is the reason of inhibitory potency in **1a**–**4a**, however, **4a** with electron donor group (Me) is the more potent inhibitor than the **3a**. Therefore this explanation was inadequate to explain the order of inhibitory and it seems other factors such as steric effect and hydrophobicity are also affect on inhibitory potency of selected compounds. The same order was also observed for **1b**–**4b**.

Hydrophobicity. It is well known that for *in vitro* (and *in vivo*) experiments with enzyme, small difference in hydrophobic character can be quite important.

The hydrophobicity of selected inhibitors with same amid moiety, decreased when chlorine groups replaced by morpholine substituents. Therefore, hydrophobicity also affect on high inhibitory potency of **1a**–**4a** relative to **1b**–**4b**.

As shown in Tables II and III, compound **2a** is the most potent inhibitor in comparison with compounds **1a**, **3a**, and **4a** and a similar result was obtained for compound **2b** relative to compounds **1b**, **3b**, and **4b**. The more inhibitory effect of compounds **2a** and **2b** on hAChE may be due to their large hydrophobicity.

The inhibitory potency of these compounds was different and varied depending on the enzyme source. The differences could be due to chemical variation between these compounds (Scheme 1), which results in distinct interactions with the two classes of cholinesterases and to structural differences between the enzymes tested (BuChE–serum or AChE–erythrocyte membrane), mainly to primary and tertiary enzyme structure peculiarities.

The dimensions and the microenvironment of the active site gorge play a significant role in determining the selectivity, affinity and hydrolysis rate of substrates and inhibitors of esterases [15,23].

In vivo *study*

The data in Table IV suggest that there is some seasonal variation in both the kinetic characteristics of AChE and BChE, and their susceptibility to inhibitors. It is not clear whether such variability arises from endogenous factors (tertiary structure) or from exogenous factors such as environmental changes (e.g., inhibitor structure).

Our *in vitro* study showed that new synthesised carbacylamidophosphates are moderate cholinesterases inhibitors, but *in vivo* experiment indicated that they have weak inhibitory activity.

The organophosphate compounds are inhibitors of serine esterases and serine proteases, and have a high affinity for cholinesterases [26]. The group among the singly bonded constituents that is connected to the phosphorus by the least stable bond is considered the "leaving group," and it is the labile group eliminated during the reaction in which the organophosphate creates a covalent bond with the catalytic serine group of the esterase or protease. Thus during this phosphorylation reaction, the organophosphate molecule is destroyed through stoichiometric reaction with the enzyme. The hydrophobicity of organophosphate compounds is an important factor in forming the phosphoryl–serine linkage both in *in vitro* and in *in vivo* environments. One such protective biochemical mechanism arises from the family of non-target serine esterases, which include the carboxylesterases, non-target AChE (such as occurs in erythrocytes), and butyrylcholinesterase [26].

Conclusion

The electronic properties of the carbacylamidophosphates such as the charge on the phosphorus atom, hydrophobicity and the leaving group connected to the phosphorus atom are the most important factors which can affect the inhibition of AChE. Some other factors such as dimensions and microenvironment of the active site gorge of the specified enzyme have an important role in inhibitor interactions with the specified enzyme. Differences in *in vitro* and *in vivo* results may be due to protection by the non-target esterases and other unknown limitations which led to weak inhibitory potency of these inhibitors on AChE activity.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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