Some New Carbacylamidophosphates as Inhibitors of Acetylcholinesterase and Butyrylcholinesterase

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The differences in the inhibition activity of organophosphorus agents are a manifestation of different molecular properties of the inhibitors involved in the interaction with the active site of enzyme. We were interested in comparing the inhibition potency of four known synthesized carbacylamidophosphates with the general formula $RC(O)NHP(O)Cl_2$, constituting organophosphorus compounds, where R = CCl_3 (**1**), $CHCl_2$ (**2**), CH_2Cl (**3**) and CF_3 (**4**), and four new ones with the general formula $RC(O)NHP(O)(R')_2$, where R′ = morpholine and R = CCl_3 (**5**), $CHCl_2$ (**6**), CH_2Cl (**7**), CF_3 (**8**), on AChE and BuChE activities. In addition, *in vitro* activities of all eight compounds on BuChE were determined. Besides, *in vivo* inhibition potency of compounds **2** and **6**, which had the highest inhibition potency among the tested compounds, was studied. The data demonstrated that compound **2** from the compound series **1** to **4** and compound **6** from the compound series **5** to **8** are the most sensitive as AChE and BuChE inhibitors, respectively. Comparing the IC_{50} values of these compounds, it was clear that the inhibition potency of these compounds for AChE are 2- to 100-fold greater than for BuChE inhibition. Comparison of the kinetics (IC_{50}, K_i, k_p, K_A and K_D) of AChE and BuChE inactivation by these compounds resulted in no significant difference for the measured variables except for compounds **2** and **6**, which appeared to be more sensitive to AChE and BuChE by significantly higher k_p and K_i values and a lower IC_{50} value in comparison with the other compounds. The LD_{50} value of compounds **2** and **6**, after oral administration, and the changes of erythrocyte AChE and plasma BuChE activities in albino mice were studied. The *in vivo* experiments, similar to the *in vitro* results, showed that compound **2** is a stronger AChE and BuChE inhibitor than the other synthesized carbacylamidophosphates. Furthermore, in this study, the importance of electropositivity of the phosphorus atom, steric hindrance and leaving group specificity were reinforced as important determinants of inhibition activity.

Key words: Carbacylamidophosphate, AChE, BuChE

Introduction

Acetylcholinesterase (AChE) is of interest to enzymologists because of its critical role in neurotransmission and its high catalytic power (Quinn, 1987; Kovach, 1988; Taylor and Radic, 1994). It is readily phosphorylated at the active serine site by a variety of organophosphorus compounds which are mainly used as insecticides (Aldrich and Reiner, 1972; Taylor, 1990). Phosphorylation of acetylcholinesterase leads to the formation of stable covalent conjugates depending on the structure of the inhibitor and the particular enzyme studied (Ordentlich *et al*., 1993; Haux *et al.*, 2000). Butyrylcholinesterase (BuChE) is very similar in structure and catalytic function to acetylcholinesterase, but have a less well-defined role in biological processes (Taylor and Radic, 1994; Massoulie *et al.*, 1993). The differences in the sensitivity of AChE from different species to *in vitro* inhibition by organophosphorus agents have been documented in numerous comparative studies (Chattopadhyay *et al.*, 1986; Johnson and Wallace, 1987; Kemp and Wallace, 1990). The chemistry of organophosphorus inhibition of AChE is well established and provides a framework for investigating species differences in the molecular properties associated with enzyme inactivation. The inhibitors associate with AChE via Coulombic forces between the electron-defection phosphorus atom and a nucleophilic centre within the esteratic subsite of the enzyme. The formation of this reversi-

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ble complex, as reflected by the dissociation constant (K_D), is limited by steric hinderance possibly reflecting the finite dimensions of the esteratic subsite of AChE (Anderson *et al.*, 1977; Mundy *et al.*, 1978). Once formed, the complex undergoes rapid nucleophilic substitution wherein the enzyme becomes irreversibly phosphorylated at its nucleophilic centre, releasing the leaving group of the respective inhibitor. The electronic properties of the phosphorus atom in distinguishing among the inhibitor potency of dissimilar organophosphorus agents are well documented (Darlington *et al.*, 1971; Hansch and Deutsch, 1966). Structure-activity correlations have been employed to establish the principal chemical properties responsible for discriminating between different organophosphorus inhibitors (Metcalf and Frederickson, 1965).

In our previous studies we discussed the synthesis, characterization, hydrolysis, and inhibition potency on human erythrocyte AChE activity of four carbacylamidophosphates with the general formula $RC(O)NHP(O)Cl_2$, and constituted organophosphorus compounds with R = CCl_3 (**1**), $CHCl_2$ (**2**), CH_2Cl (**3**) and CF_3 (**4**). Based on spectroscopic data and hydrophobicity evaluation we considered the electronic properties of the phosphorus atom and the hydrophobicity of the surrounding substituents on the hydrolysis tendency and the inhibitory potency of the selected compounds (Gholivand *et al.*, 2006).

Since the differences in the inhibition potency of organophosphorus agents are a manifestation of differing molecular properties of the inhibitors involved in the interaction with the active site of an enzyme, we were interested in studing the inhibition potency of four new synthesized carbacylamidophosphates (Gholivand *et al.*, 2007) with the general formula $RC(O)NHP(O)(R')_2$, where R′ = morpholine and R = CCl_3 (**5**), $CHCl_2$ (**6**), CH_2Cl (**7**), CF_3 (**8**), on AChE and BuChE activities and in comparing them with the inhibition activity of compounds **1**–**4** (Fig. 1). In addition, *in vitro* activities of **1**–**8** on BuChE and AChE and their kinetic parameters, inhibitory potency (IC_{50}), bimolecular rate constants (K_i), dissociation constant (K_D) and phosphorlation constant (k_p), were determined. Besides, *in vivo* IC_{50} inhibition potency of compounds **2** and **6**, the most potent inhibitors, on AChE and BuChE activity and their acute toxicity (LD_{50}) were studied.

Materials and Methods

Chemicals

All syntheses of compounds **1**–**8** based on published methods (Gholivand *et al.*, 2006, 2007) and were carried out under an argon atmosphere. Purified human plasma AChE (3.1.1.7; 50 units/785 μl), purified horse plasma BuChE (lyophilized) from Sigma-Aldrich (UK), butyrylthiocholine (BuTCh) iodide, acetylthiocholine (ATCh) iodide, and 5, 4′-dithio-bis(2-nitrobenzoic acid) (DTNB) from Fluka (Tehran, Iran) were used. All other chemicals and solvents were purchased from Merck (Tehran, Iran).

AChE activity assay

The activity of AChE was determined by a modified Ellman method (Ellman *et al.*, 1961): The

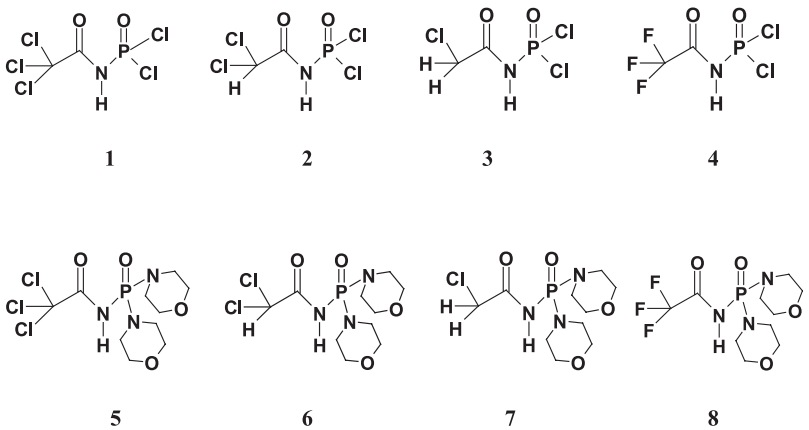


Fig. 1. Structures of compounds **1**–**8**.

level of ATCh hydrolysis was monitored by liberated thiocholine which 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 ATCh (0.135 mM final concentration, 15 μl). The absorbance change at 37 °C was monitored with a 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 as the AChE activity by measuring thiocholine which reacted with DTNB after hydrolysis of BuTCh. The lyophilized BuChE was diluted with 100 mM phosphate buffer (pH 8) for the activity assay.

AChE and BuChE inhibition

The reaction mixtures for the determination of IC_{50} values of human erythrocyte AChE consisted of a DTNB solution (5 μl), inhibitors (4.5, 9.5, 25, 47, 62 and 72 μl), ATCh iodide solution (10 μl), and phosphate buffer (pH 7.4) in a final volume of 100 μl. The final concentrations of DTNB and ATCh were 10^{-4} and $2.7 \cdot 10^{-5}$ M. The initial concentrations of inhibitors in ethanol were $3.57 \cdot 10^{-5}$, $4.08 \cdot 10^{-5}$, $4.76 \cdot 10^{-5}$, $4.38 \cdot 10^{-5}$, $2.62 \cdot 10^{-5}$, $2.89 \cdot 10^{-5}$, $3.21 \cdot 10^{-5}$, and $3.02 \cdot 10^{-5}$ M, respectively. The enzyme concentration in the assay was $1.004 \cdot 10^{-10}$ M. The reaction mixtures for the IC_{50} values determination of BuChE were the same as in the AChE assay but the volumes of inhibitors were 5.4, 10.5, 28, 48, 65, and 76 μl. The candidate inhibitors were incubated with the enzyme (AChE or BuChE), inhibitors, and DTNB for 5–10 min at 37 °C prior to the addition of ATCh or BuTCh for the residual activity assay. The samples were placed in 96-well ELISA dishes (Nunc, Denmark) and the results were obtained by placing the 96-well dishes into an ELISA plate reader (Stat-Fax 303 plus, Awareness Technology Inc, Palm City, FL, USA) at 412 nm. The data were collected every 4 min for 24 min.

Calculation of hydrophobicity

Calculated hydrophobicity extent, log *P*, of the eight synthesized compounds was performed using the software log*P* (ChemDraw Ultra, 8. 0.3, 2003).

Animals

NMRI albino mice, weighing 20–25 g, were obtained from Pharmaceutical Sciences Research Center (PSRC) of Tehran University of Medical Sciences (TUMS), Tehran, Iran. The animals were all individually housed in plastic cages in an air-conditioned room with controlled temperature (20–22 °C), automatic lighting and free access to standard laboratory diet and water. Animals were maintained under this conditions for 10 d prior to the experiment. They were randomly divided into three groups consisting of four mice each. Regarding the *in vitro* inhibitory concentration (IC_{50}) of paraoxon in human erythrocytes [(4.7 ± 0.9) μM] (Gupta, 2006) as the reference compound, the equimolar dose and two upper and lower doses of compounds **2** and **6** in an 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 compounds **2** and **6** 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 benzethonium chloride was determined by measuring the maximum absorbance at 440 nm with a double beam spectrophotometer. 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 (George and Abernethy, 1983).

BuChE activity in plasma

10 μl 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 BuTCh iodide were added to the sample tube, and the change in the absorbance was measured at 412 nm using a double beam spectrophotometer (Ellman *et al.*, 1961).

Determination of toxicity (LD_{50})

In order to determine the acute toxicity (LD_{50}) of compounds **2** and **6**, various doses [100, 300,

600, 1000, 1500, and 2000 mg/(kg · day)] were gavaged to NMRI albino mice in separate groups of 4 each. The animals were observed for 48 h and any mortality was recorded at the end of this period. The LD_{50} value was determined by regression probit using Stats Direct (Chan and Hayes, 1989).

Results

The derivation of the kinetic values is based on the works of Kitz and Wilson (1962) and Segel (1975). The reaction describing the inhibition process and the definition of the kinetic parameters is based on the following scheme:

$$[E] + [I] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [EI]_R \xrightarrow{k_p} [EI]_I,$$

where [E] and [I] represent the concentration of free enzyme and inhibitor, respectively, $[EI]_R$ is the reversible enzyme-inhibitor complex, and $[EI]_I$ the irreversibly phosphorylated enzyme-inhibitor complex; k_p is the rate constant of AChE phosphorylation and K_i the bimolecular inhibition constant with

$$K_m = k_{-1} + k_p/k_1.$$

Assuming that $k_p >> k_{-1}$, then

$$K_m = k_{-1}/k_1 = K_D = 1/K_A,$$

$$K_{eq} = k_1 k_p/k_{-1} = k_p/K_D = K_A \times k_P = K_i,$$

where K_A is the association constant and K_D the dissociation constant.

The fraction of AChE activity remaining at time t was calculated by dividing the remaining AChE activity by the original uninhibited enzyme activity ($[EI]_t/[EI]_o$). Least squares linear regression (ln) of the fraction of remaining AChE activity ($[EI]_t/[EI]_o$) versus time (in min) resulted in a line at each inhibitor concentration with the slope $-k_{app}$

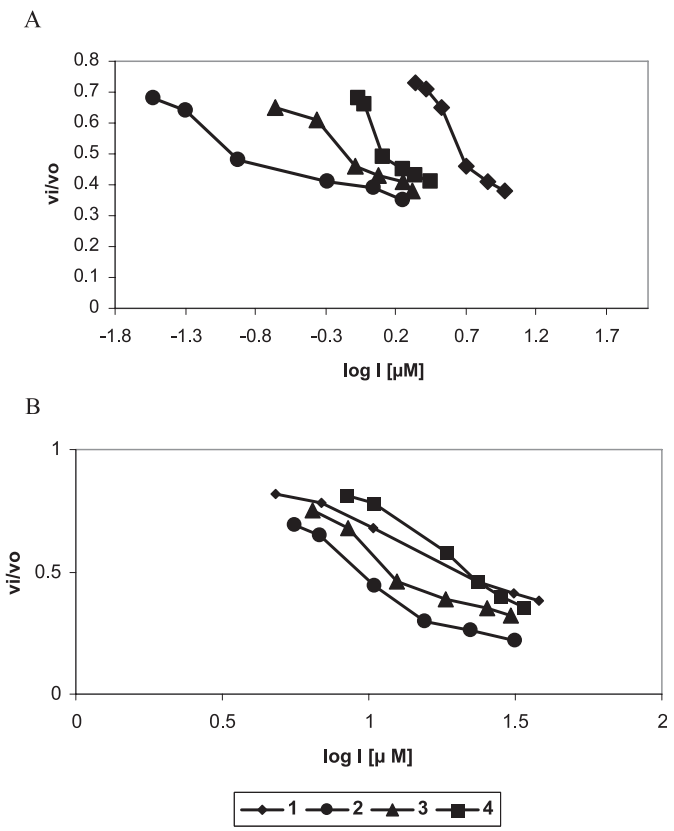


Fig. 2. Plot of v_i/v_o against log [I] of human erythrocyte AChE (A) and of BuChE (B) for inhibitors **1**–**4**. v_i and v_o are the activity of enzyme in the presence and absence of inhibitor, respectively, and [I] is the inhibitor concentration (in μM).

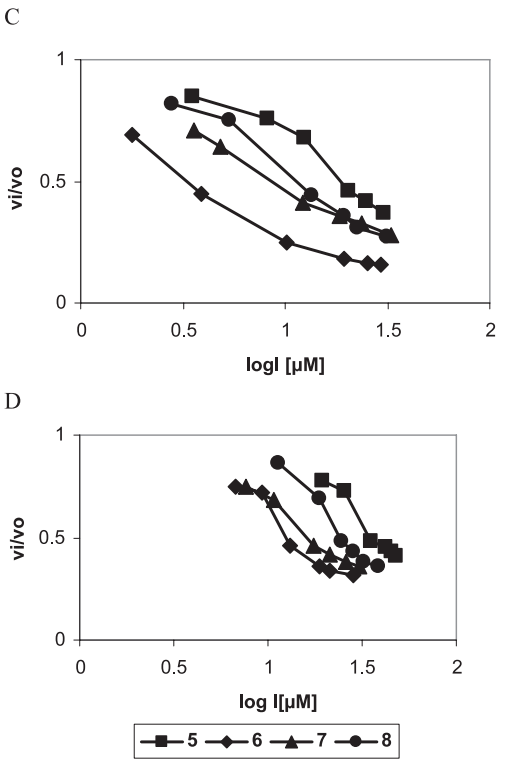


Fig. 3. Plot of v_i/v_o against log [I] of human erythrocyte AChE (C) and of BuChE (D) for inhibitors **5**–**8**. v_i and v_o are the activity of enzyme in the presence and absence of inhibitor, respectively, and [I] is the inhibitor concentration (in μM).

(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 = $-1/K_D = -K_A$ (Kitz and Wilson, 1962).

The *in vitro* inhibition of AChE and BuChE for compounds **1**–**4** are presented in Fig. 2. Fig 3 represents the *in vitro* inhibition of AChE and BuChE for compounds **5**–**8**, and the IC_{50} values are included in Table I. The data demonstrate that compound **2** from compound series **1**–**4** and compound **6** from compound series of **5**–**8** were the most sensitive as AChE and BuChE inhibitors. By comparing the IC_{50} values of these 8 compounds, it is clear that the inhibition potency of these compounds in AChE was nearly 2- to 100-fold higher than the BuChE inhibition. Furthermore, compounds **1**–**4** had a stronger inhibitory effect on both AChE and BuChE than compounds **5**–**8**. A representative set of data for determining k_{app} at six concentrations of compound **6** is presented in Fig. 4. Least squares linear regression of the data at each inhibitor concentration resulted in a line having the slope $-k_{app}$ with regression coefficients between 0.98 to 0.99. As the concentration of compound **6** increased, the slope increased, resulting in larger k_{app} values. A double reciprocal plot of the concentration of compound **6** versus the k_{app} values from Fig. 3 resulted in a line with slope = $1/K_i$, y-intercept = $1/k_p$ and x-intercept = $-1/K_D = -K_A$ and regression coefficients between 0.98 to 0.99 (Fig. 5). The mean K_i, k_p, K_A and K_D values for all eight compounds were calculated for AChE and BuChE and are summarized in Table I. These data suggest that the inhibition of AChE and BuChE follows a pseudo-first-order Michaelis-Menten process. Comparison of the kinetics (IC_{50}, K_i, k_p, K_A and K_D) of AChE and BuChE inactivation by these eight compounds resulted in no significant difference for the measured variables except for compounds **2** and **6**, which ap-

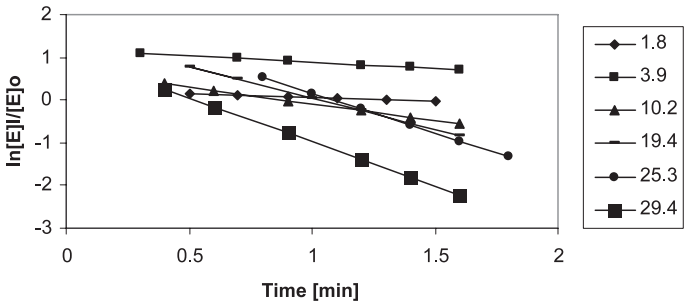


Fig. 4. A representative set of data for determining $-k_{app}$ at six concentrations of **6** for human erythrocyte AChE. The time of incubation was variable for each inhibitor concentration and the slope of the lines ($-k_{app}$) increased with increasing inhibitor concentration. Compound **6** concentrations were: 1.8, 3.9, 10.2, 19.4, 25.3, and 29.4 μM.

Table I. Kinetics of acetylcholinesterase inactivation by compounds **1**–**4** (A) and **5**–**8** (B), respectively, and kinetics of butyrylcholinesterase inactivation by compounds **1**–**4** (C) and **5**–**8** (D), respectively.

A

Compound	IC_{50} [μM]	K_i [mmol min]$^{-1}$	k_p [min^{-1}]	K_A [mM^{-1}]	K_D [μM]
1	3.8 ± 0.4[a]	140 ± 20	10.92 ± 2.4	17.1 ± 3.1	75.8 ± 4.38
2	0.052 ± 0.02	980 ± 40	47.8 ± 13.6	38.1 ± 10.7	41.2 ± 4.71
3	0.63 ± 0.12	760 ± 30	37.7 ± 9.3	31 ± 5.3	48.6 ± 9.69
4	2.4 ± 1.19	450 ± 40	23.4 ± 3.6	27 ± 6.6	52.3 ± 3.28

B

Compound	IC_{50} [μM]	K_i [mmol min]$^{-1}$	k_p [min^{-1}]	K_A [mM^{-1}]	K_D [μM]
5	27.7 ± 8.2[a]	20.4 ± 9.7	5.83 ± 1.5	3.5 ± 1.04	452.6 ± 46.1
6	3.15 ± 0.85	93 ± 11	18.8 ± 3.4	6.8 ± 1.48	195.6 ± 7.64
7	5.81 ± 0.61	78 ± 13	10.9 ± 2.6	5.2 ± 0.4	223.8 ± 15.9
8	15.4 ± 3.8	46 ± 14	12.9 ± 2.3	4.2 ± 1.1	368.2 ± 35.9

C

Compound	IC_{50} [μM]	K_i [mmol min]$^{-1}$	k_p [min^{-1}]	K_A [mM^{-1}]	K_D [μM]
1	30.5 ± 5.3[a]	1.8 ± 0.3	4.4 ± 1.1	0.55 ± 0.01	2228.3 ± 11
2	7.3 ± 1.6	9.8 ± 0.6	16.4 ± 2.1	0.752 ± 0.11	1623.2 ± 67.7
3	13.1 ± 1.9	8.2 ± 0.9	13.3 ± 1.9	0.68 ± 0.076	1723 ± 67.4
4	22 ± 2.6	6.2 ± 0.6	11.5 ± 1.4	0.592 ± 0.04	1862 ± 57

D

Compound	IC_{50} [μM]	K_i [mmol min]$^{-1}$	k_p [min^{-1}]	K_A [mM^{-1}]	K_D [μM]
5	49.4 ± 14.3[a]	0.14 ± 0.03	2.1 ± 0.7	0.046 ± 0.006	25635.2 ± 194.2
6	13.7 ± 1.2	0.82 ± 0.04	12.3 ± 1.1	0.074 ± 0.005	14832.2 ± 339.5
7	17.9 ± 1.3	0.68 ± 0.064	10.2 ± 0.7	0.079 ± 0.015	15823.1 ± 198.1
8	25.1 ± 1.9	0.46 ± 0.08	7.6 ± 1.1	0.062 ± 0.004	17352.3 ± 110.9

[a] Values represent the means ± SD of three individual parameter determinations.

Table II. Data resulted from *in vivo* experiments.

Compound	LD_{50} [mg/kg]	AChE IC_{50} [μM]	BuChE IC_{50} [μM]
2	125 ± 12.2[a]	110 ± 8.2	1096 ± 14.3
6	190 ± 14.7	375 ± 11.6	1263 ± 21.2

[a] Values represent the means ± SD of three individual parameter determinations.

peared to be more sensitive to AChE and BuChE expressed by the significantly greater k_p and K_i values and the lower IC_{50} values.

As shown in Table I, parts A and C, the AChE IC_{50} value of compound **2** is nearly 12- to 70-fold greater than of compounds **1**, **3**, and **4**. Also, comparison between the AChE IC_{50} values of compound **6** with **5**, **7**, and **8** shows the smaller difference ranges, from 2- to 9-fold, in inhibition activity.

These differences are also evident from the nearly 5- to 7-fold greater K_i values for compounds **2** and **6** relative to the other compounds, which result in both larger phosphorylation rate constants (k_p) and K_A values.

The acute toxicity experiment (LD_{50}) demonstrated that compounds **2** and **6** are lethal up to a dose of <600 mg/kg after oral administration (Table II). Treatment with the compounds **2** and **6** by doses of <100 mg/(kg · day) inhibited both AChE activity in erythrocytes and BuChE activity in

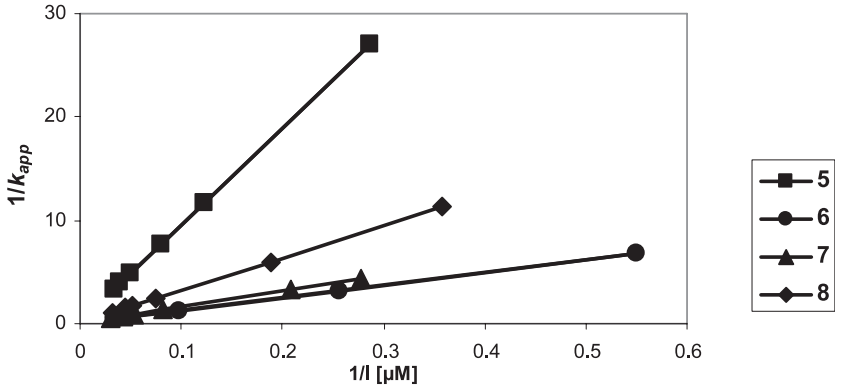


Fig. 5. A representative double reciprocal plot of inhibitor concentrations versus k_{app}. Linear regression of six points representing k_{app} at six inhibitor concentrations for each inhibitor resulted in a line with slope $1/K_i$, y-intercept = $1/k_p$, and x-intercept = $-1/K_D = -K_A$.

Table III. IR, δ (^{31}P) and calculated hydrophobicity data for compounds **1** to **8**.

Compound	M_W [g/mol]	Clog P	δ (^{31}P) (ppm)	(P=O) [cm^{-1}]	ν(P–Cl) [cm^{-1}]	ν(P–N$_{amine}$) [cm^{-1}]
1	279.3	0.397	8.08	1181	590	–
2	244.8	–0.306	8.20	1194	580	–
3	210.4	–1.36	6.62	1125	585	–
4	229.9	–0.923	7.42	1180	593	–
5	380.6	2.917	9.51	1197	–	1108
6	346.1	2.214	9.58	1199	–	1106
7	311.5	1.151	8.39	1173	–	1108
8	331	1.597	8.44	1193	–	1094

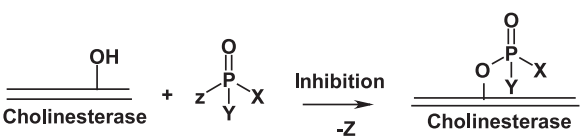
plasma (Table II). Compound **2** had a higher inhibition activity in AChE (IC_{50} = 110 μM) than compound **6**.

Discussion

The initial alteration at the active site of the enzyme may involve local changes in the peptide conformation and charge or steric repulsion imposed by the phosphoryl group. Structure-activity correlations have been employed to establish the principal chemical properties responsible for discriminating between different organophosphorus inhibitors (Metcalf and Frederickson, 1965). The inhibition process is dependent upon the reactivity, stereochemistry and leaving group in organophosphorus compounds (Thompson *et al.*, 1996). The importance of the electropositivity of the phosphorus atom in the Coulombic association of the inhibitors with AChE is well known. As we discussed in our previous work (Gholivand *et al.*, 2006) and according to data presented in Table III, ^{31}P chemical shifts for the synthesized compounds revealed that the phosphorus atom in compounds **1**–**4** has less electropositivity than in compounds **5**–**8**. Comparing the infrared vibrational frequencies for the P=O bond demonstrated that this bond is tighter in compounds **5**–**8** than in **1**–**4**. It might be expected that the inhibition potency of compounds **5**–**8** should be larger than of **1**–**4**. Conversely, as shown in Table I, parts A and C, the IC_{50} values of the above mentioned compounds are nearly 7- to 60-fold less than of compounds **1**–**4**. It can be resulted that some other structural factors affect the inhibition potency of these organophosphorus compounds on AChE. Steric exclusion of bulky inhibitors from the active site has been implicated as a major factor governing the inhibition of AChE (Fukuto *et al.*, 1959). Consequently, it has been proposed that resistance to organophosphorus may be conferred, in part, by the limiting dimensions of the esteratic subsite of AChE (Kemp and Wallace, 1990). The calculated log P (O/W) values as a measure of hydrophobicity for all eight synthesized inhibitors are pre-

sented in Table III. Despite the fact that the heterocyclic ring, morpholine, in compounds **5**–**8** adopts a chair conformation as the most stable conformer, similar to that of cyclohexane, to relieve steric strain, and is slightly flattened at the nitrogen and oxygen ends (Gholivand *et al*., 2007), the calculated log *P* (O/W) values of these compounds are greater than those for compounds **1**–**4**. By comparing the IC_{50} values of inhibitors for AChE, it was demonstrated that compounds **5**–**8** with more hydrophobic character have less inhibition potency on AChE than compounds **1**–**4**. It was revealed that, although the phosphorus atom of compounds **5**–**8** is more electropositive relative to compounds **1**–**4**, hydrophobic subsistents hinder rather than enhance the association of organophosphorus with AChE. This can also be discussed by comparing the low affinity (large K_D) of AChE to the inhibitors **5–8** relative to compounds **1**–**4**.

To be a potent inhibitor of cholinesterase, an organophosphorus compound usually contains a good leaving group Z (Scheme 1), to form of a phosphoserine linkage (Thompson *et al*., 1996). Replacing the chlorine atoms in **1**–**4** with morpholine (compounds **5**–**8**) resulted in a smaller inhibitory effect on AChE (Table I, parts A and C). The crystallographic data for compounds with morpholine groups (Gholivand *et al*., 2007) indicated that the $P-N_{amine}$ bond lengths are between 1.63–1.64 Å, which are shorter than a P–N single bond (1.77 Å) and longer than a P=N double bond (1.57 Å) (Thompson *et al*., 1996; Corbridge, 1995). As it is known, halogen atoms are good leaving groups; therefore the P–Cl bond is more labile than the $P-N_{amine}$ bond. This leads to the easier formation of a phosphoserine linkage in the enzyme active site. In spite of the fact that replacing chlorine atoms with morpholine groups leads to more electropositivity of the phosphorus atom, increasing in hydrophobicity and bonding energy of the $P-N_{amine}$ bond are two important factors which make them unsuitable leaving groups and decrease the inhibitory potency of them on the AChE activity. The more inhibitory effect of compounds **2** and **6** on AChE, in spite of large hydrophobicity may be due to their more electropositive of phosphorus atom expressed by ^{31}P chemical shifts (Table III).



Scheme 1. Proposed mechanism for AChE and BuChE inhibition by organophosphorus compounds.

By comparing data of Table I, parts B and D with parts A and C, it is clear that the inhibitory effects of **1**–**8** are nearly 2- to 100-fold higher on AChE than on BuChE. X-Ray structures of AChE from various sources showed that the catalytic site is located near the bottom of a deep and narrow "gorge" (Sussman *et al.*, 1991; Kryger *et al*., 2000). One of the striking features of this gorge is related to the presence of 14 aromatic residues, which line about 40% of its surface and which are highly conserved in enzymes from different species (Axelsen *et al*., 1994). Yet, BuChE, another type of cholinesterase found in vertebrates, catalyzes acetylcholine hydrolysis as efficiently as AChE, although six of the active site gorge aromatic residues are replaced by aliphatic amino acids (Cygler *et al*., 1993). According to early hypothesis and modeling experiments (Jarv, 1984), the main functional differences between the AChE and BuChE active sites are related to the structure of the acyl pocket. Accordingly, BuChE is more reactive than AChE toward bulky substrates such as DFP or paraoxon as organophosphorus inhibitors. The latest results based mainly on determining the activity of AChE and BuChE toward certain stereoselective alkylphosphorates implying that active centres of AChE and BuChE may not be as similar as thought before (Ordentlich *et al*., 1999; Quistad *et al*., 2005). It is rather surprising to find that compounds **5**–**8** with more hydrophobicity due to morpholine bulky groups have nearly 2-fold less IC_{50} values than compounds **1**–**4** (Table I, parts B and D).

The *in vivo* toxicological studies involve several aspects for the carbacylamidophosphates. Albino mice were used as a model for the *in vivo* study of AChE and BChE inhibition. This system has several advantages; firstly, Albino mice erythrocytes and plasma are a convenient AChE and BuChE source with high activity and sensitvitey to organophosphorus inhibitors. Secondly, *in vivo* inhibition of enzymatic activity correlates with poisoning signs and toxicity for carbacylamidophosphate. Due to these advantages, the *in vivo* AChE and BuChE activities of the most potent inhibitors **2** and **6** were determined, and the albino mice cholinesterase activity from control group and from

the animals of the *in vivo* study after treatment for 24 h are shown in Table II.

The results demonstrate that the inhibitory potency of selected compounds was different depending on the enzyme source. It may be due to the different tertiary structure of the enzyme source or arises from the different structure of the inhibitors.

In addition, an *in vitro* study showed that new synthesized carbacylamidophosphates are moderate cholinesterase inhibitors, but an *in vivo* experiment indicated that they have weak inhibition activity. As carbacylamidophosphate compounds can react with a family of target and nontarget serine esterases and nontarget serine proteases, hydrolysis of these compounds by nontarget esterases and unknown limitation probably cause that these inhibitors have very weak inhibitory activity on cholinesterases in *in vivo* studies.

In summary, comparison of reactivity profiles of compounds **1**–**8** suggests that the molecules with the core unit –C(O)NHPO)– are weak inhibitors to both AChE and BuChE in comparison with strong inhibitors like paraoxon. The IC_{50} values of erythrocytes in AChE and the LD_{50} values in oral administration of compounds **2** and **6** demonstrated that paraoxon is nearly 70-fold more toxic and a stronger inhibitor than **2** and **6**. Also the results show that they are not proper inhibitors for acting with the active site of the BuChE relative to the AChE enzyme. Furthermore, the importance of electropositivity of phosphorus atom, steric hindrances and leaving group specificity are reinforced as important determinants of inhibition potency. However, the significance of each of these factors differs between compounds based on their chemical structures.

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