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Comparative Sensitivity of Human Acetylcholinesterase to *in vitro* Inhibition by Synthetic Analogues of Phosphoramidate Compounds

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Using phosphoryl chloride as a substrate, some phosphoramidate derivatives with the general formula $(R_1)(O)PCl(R_2)$; [R_1 = NMe_2, OC_6H_5, $OC_6H_4CH_3$ and R_2 = $N(CH_2C_6H_5)(CH_3)$, $N(CH_2C_6H_5)(C_2H_5)$] was prepared and characterized by ^1H, ^{31}P and ^{13}C NMR and IR spectroscopy and elemental analysis. Biochemical studies conducted to evaluation of sensitivities of human acetylcholinesterase to inhibition of these compounds. Determination of human erythrocyte acetylcholinesterase (hAChE) activity was carried out according to the Ellman's modified kinetic method. Biomolecular rate constant (k_i) of the selected compounds towards the active site of hAChE ranged between 2.4×10^{-3} and 5.23×10^{-1} M^{-1} min^{-1} and their IC_{50} values varied from 2.33×10^{-1} to 1.99×10^{-3} mM. The difference in the inhibitory potency of these compounds is discussed with respect to their hydrophobicity. A comparison of the k_i and IC_{50} values for inhibition of hAChE by these inhibitors revealed that hydrophobic, steric and electronic factor of phosphorus substituents are important in potency of inhibitory on hAChE.

Key Words: Ellman's method, Hydrophobicity, Human acetylcholinesterase, IC_{50}, Inhibitor, k_i, Phosphoramidate.

INTRODUCTION

Activation of the human complements system, a major line of defense against infection (viruses, bacteria, fungi and certain parasites), require the participation of serine esterases such as acetylcholinesterase (AChE)[1]. The widely used anticholinesterase insecticides such as phosphoramidate compounds inhibit AChE by phosphorylation of a serine hydroxyl group in the serine active site, located at the bottom of a deep narrow gorge[2-6]. Phosphoramidates and structurally related compounds have been subject

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of intensive study for more than a century[7,8]. In the course of structure-activity studies on AChE inhibitors and based on previous finding, it has been obvious that the inhibitory potency of organophosphorus compounds is dependent upon hydrophobicity, steric and electronic parameters. However, the preparation in high yield of them that has activity against a broad range of insects and low mammalian toxicity, is considerable interest. In our previous works[9] we discussed the synthesis, characterization, hydrolysis and inhibition potency on human erythrocyte acetylcholinesterase activity of novel thiono and seleno phosphoramidate compounds with low reactivity toward hAChE. Based on spectroscopic data and hydrophobicity evaluation we considered the differences in hydrophobicity of thiono and seleno analogues of phosphoramidate compounds with identical leaving group may be important than their electronic differences in determining their effectiveness as hAChE inhibitors[9].

The present study evaluates inhibitory potency of six phosphoramidate compounds on human acetylcholinesterase (hAChE). This information will be used for the comparison of effects characteristic to the three sets of structural parameters involved in the toxic actions of these compounds: (1) variation in the P-substituents bonds of P-O and P-N, (2) variation in the aromatic esters of P-OC_6H_5 and P-$OC_6H_4CH_3$ and (3) variation in the amine group of N-CH_3 and N-C_2H_5. In addition, information is provide on variation toxicity between species for closely related compounds. The information which is not permitting conclusions to be drawn at this time, may provide the basis for further studies in pharmacology and/or enzymology. A detailed understanding of these effects will serves as the basis for rational design of compounds with specific and predictable properties. Herein, four novel phosphoramidates $(CH_3)_2NPOClN(CH_2C_6H_5)(CH_3)$ (**1a**), $(CH_3)_2NPOClN(CH_2C_6H_5)(C_2H_5)$ (**2a**), $(CH_3)(C_6H_5CH_2)NP(O)(OC_6H_5)Cl$ (**1c**) and $(C_2H_5)(C_6H_5CH_2)NP(O)(OC_6H_5)Cl$ (**2c**) were synthesized and characterized by ^1H, ^{13}C, ^{31}P NMR and IR spectroscopy and elemental analysis and two phosphoramidate $(CH_3)(C_6H_5CH_2)NP(O)(OC_6H_4CH_3)Cl$ (**1b**), $(CH_3)(C_6H_5CH_2)NP(O)(OC_6H_4CH_3)Cl$ (**2b**) were synthesized according to literature method[10]. *In vitro* activity of hAChE after inhibition by six phosphoromidates was measured and its kinetic parameters (inhibitory potency; IC_{50} and biomolecular rate constants; k_i) were determined. We also simultaneously performed experimental determination of hydrophobicity of these compounds.

EXPERIMENTAL

^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker 250 MHz spectrometer. ^1H, ^{13}C and ^{31}P chemical shifts were determined relative to TMS and 85 % H_3PO_4, respectively, as external standards. IR spectra (KBr plate and liquid) were obtained with a Bruker, Tensor 27 model spectrometer.

UV measurements were performed by Unicam 8700 spectrophotometer. GC spectra were obtained with Agilent 6890 GC column.

Acetylthiocholine (ASCh), 5,5′-dithio*bis*(2-nitrobenzoic acid) (DTNB) and human acetylcholinesterase (hAChE); EC 3.1.1.7) were obtained from Sigma Chemical Co. and used directly without further purification. Human acetylcholinesteras was diluted to 1:25 time in a phosphate buffer (Na_2HPO_4/NaH_2PO_4), 70 mM, pH = 7.4). All other reagents for enzymatic experiments were purchased from Fluka. All the chemical and solvents for synthesis were taken from Merck.

Synthesis

$(CH_3)_2NP(O)Cl_2$ (A): Synthesized and purified using reported method[11].

General method (1a and 2a): A 100 mL round-bottom flask was charged with 4.12 mmol of nucleophiles (R_2NH), in 60 mL of benzene and 0.41 g (4.12 mmol) of Et_3N and solution was cooled to 5 °C, **A** (1.32 g, 4.13 mmol) was added slowly to the solution. Mixture of reaction was stirred at 5 °C for 1-2 h. The solution was then heated with a heating mantle and was refluxed for 1 h. The solution was filtered and volatiles were removed and the remaining solid was purified by column chromatography. (SiO_2, hexane/ethyl acetate, 2/1 as eluents).

$(CH_3)_2NP(O)Cl(N(CH_2C_6H_5)(CH_3)$ (1a): Yield, 81 %; Anal. calcd. (%) for $C_{10}H_{16}N_2OPCl$ (246.68): C, 48.69; H, 6.54; N, 11.36. Found: C, 48.68; H, 6.55; N, 11.36. $^{31}P\{^1H\}$NMR ($CDCl_3$) δ = 9.66. ^1H NMR: δ; 2.58 (3H, d, $^3J_{P\text{-}H}$ = 11 Hz, CH_3-N'), 2.74 (6H, d, $^3J_{P\text{-}H}$ = 13.01 Hz, CH_3-N), 4.25 (4H, q, $^3J_{P\text{-}H}$ = 11.95, CH_2-Ph), 7.28-7.42 (5H, m, H-Ph). ^{13}C NMR: δ; 33.19 (d, $^2J_{P\text{-}C}$ = 3.47 Hz, CH_3-N), 36.36 (d, $^2J_{P\text{-}C}$ = 2.88 Hz, CH_3-N), 52.56 (d, $^2J_{P\text{-}C}$ = 4.02 Hz, CH_2-Ph), 127.47, 128.26, 128.49, 137.32, (d, $^3J_{P\text{-}C}$ = 5.4 Hz, *ipso*-C of Ph). IR (KBr, ν_{max}, cm^{-1}): 594 m (P-Cl); 701 s, 740 s (P-N); 1240 s (P=O).

$(CH_3)_2NP(O)ClN(CH_2C_6H_5)(C_2H_5)$ (2a): Yield, 79 %; Anal. calcd. (%) for $C_{11}H_{18}N_2OPCl$ (260.71): C, 50.68; H, 6.98; N, 10.75. Found: C, 50.67; H, 6.99; N, 10.74. $^{31}P\{^1H\}$NMR ($CDCl_3$) δ = 9.45. ^1H NMR: δ; 1.11 (3H, t, $^3J_{H\text{-}H}$ = 7.1 Hz, CH_3-CH_2), 2.74 (6H, d, $^3J_{P\text{-}H}$ = 13.13 Hz, CH_3-N), 3.06 (2H, m, CH_2-N), 4.31 (2H, m, CH_2-Ph), 7.26-7.40 (5H, m, H-Ph). ^{13}C NMR: δ; 12.67 (d, $^3J_{P\text{-}C}$ = 2.35 Hz, $^{13}CH_3$-CH_2-N), 37.22 (d, $^2J_{P\text{-}C}$ = 3.02 Hz, CH_3-N), 40.32 (d, $^2J_{P\text{-}C}$ = 3.65 Hz, CH_2-N), 48.85 (d, $^2J_{P\text{-}C}$ = 4.5 Hz, CH_2-Ph), 127.566, 128.24, 128.591, 136.88 (d, $^3J_{P\text{-}C}$ = 4.9 Hz, *ipso*-C of Ph). IR (KBr, ν_{max}, cm^{-1}): 596 m (P-Cl); 702 s, 734 s (P-N); 1243 s (P=O).

$(CH_3C_6H_4O)NP(O)Cl_2$ (B), $(CH_3)(C_6H_5CH_2)NP(O)(OC_6H_4CH_3)Cl$ (1b), $(CH_3)(C_6H_5CH_2)NP(O)(OC_6H_4CH_3)Cl$ (2b) and $(C_6H_5O)P(O)Cl_2$ (C): Synthesized and purified using reported method[10].

General method (1c and 2c): A 100 mL round-bottom flask was charged with 4.12 mmol of nucleophiles (R_2NH), 60 mL of benzene and 0.41 g (4.12 mmol) of Et_3N and solution was cold to 5 °C, **C** (1.32 g, 4.13 mmol) was added slowly to the solution. Mixture of reaction was stirred at 5 °C for 1-2 h. The solution was then heated with a heating mantle and was refluxed for 1 h. The solution was filtered and volatiles were removed and the remaining solid was purified by column chromatography. (SiO_2, hexane/ethyl acetate, 2/1 as eluents).

(C_6H_5O)P(O)ClN(CH_3)($C_6H_5CH_2$) (1c): Yield, 82 %; Anal. calcd. (%) for $C_{14}H_{15}NO_2PCl$ (295.71): C, 56.87; H, 5.11; N, 4.74. Found: C, 56.88; H, 5.12; N, 4.75. $^{31}P\{^1H\}$NMR ($CDCl_3$) δ = 13.93. ^1H NMR: δ; 2.72 (3H, d, *J* = 13.26 Hz, NCH_3), 4.35 (2H, d, *J* = 10.5 Hz, CH_2Ph), 7.27-7.38 (10H, m, H-Ph). ^{13}C NMR: δ; 33.37 (d, $^2J_{P\text{-}C}$ = 3.77 Hz, CH_3-N), 53.06 (d, $^2J_{P\text{-}C}$ = 4.40 Hz, CH_2-N), 120.55 (d, $^3J_{P\text{-}C}$ = 5.31 Hz,), 125.88 (d, $^5J_{P\text{-}C}$ = 1.53 Hz,), 127.89 (s, ^{10}C); 128.33, 128.68, 129.92, 135.98, 150.01. IR (KBr, ν_{max}, cm^{-1}): 601 m (P-Cl), 725 s (P-N), 941 s (P-O), 1272 s (P=O).

(C_6H_5O)P(O)ClN(C_2H_5)($C_6H_5CH_2$) (2c): Yield, 90 %; Anal. calcd. (%) for $C_{15}H_{17}NO_2PCl$ (309.74): C, 58.17; H, 5.53; N, 4.52. Found: C, 58.17; H, 5.54; N, 4.51. $^{31}P\{^1H\}$NMR ($CDCl_3$) δ = 13.74. ^1H NMR: δ; 1.16 (t, $^3J_{H\text{-}H}$ = 7.13 Hz, C-CH_3), 3.23 (m, N-CH_2-C-), 4.42 (m, CH_2Ph), 7.24-7.37 (m, H-Ph). ^{13}C NMR: δ; 12.60 (d, $^3J_{P\text{-}C}$ = 2.52 Hz, $^{13}CH_3$-CH_2-N); 40.36 (d, $^2J_{P\text{-}C}$ = 3.77 Hz, CH_3-$^{13}CH_2$-N); 49.00 (d, $^2J_{P\text{-}C}$ = 5.03 Hz, CH_2-N); 120.63, 125.82, 127.82, 128.30, 128.66, 129.89, 136.29, 150.10. IR (KBr, ν_{max}, cm^{-1}): 603 m (P-Cl); 724 s (P-N); 939 s (P-O); 1278 s (P=O).

Kinetic studies

Acetylcholinesterase assay and inhibition experiments measurments: Activity of the enzyme was measured at 25 °C by a modified Ellman's method[12]. The reaction mixture for determination of IC_{50} values consisted of: 20 µL of DTNB solution (3.11×10^{-4} M); X µL of Inhibitor (different concentrations); 10 µL of acetylthiocholine solution (2×10^{-3} M); (950-X) µL of phosphate buffer; 20 µL of hAChE solution. The plot of V_i/V_o (V_i and V_o are the activity 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.

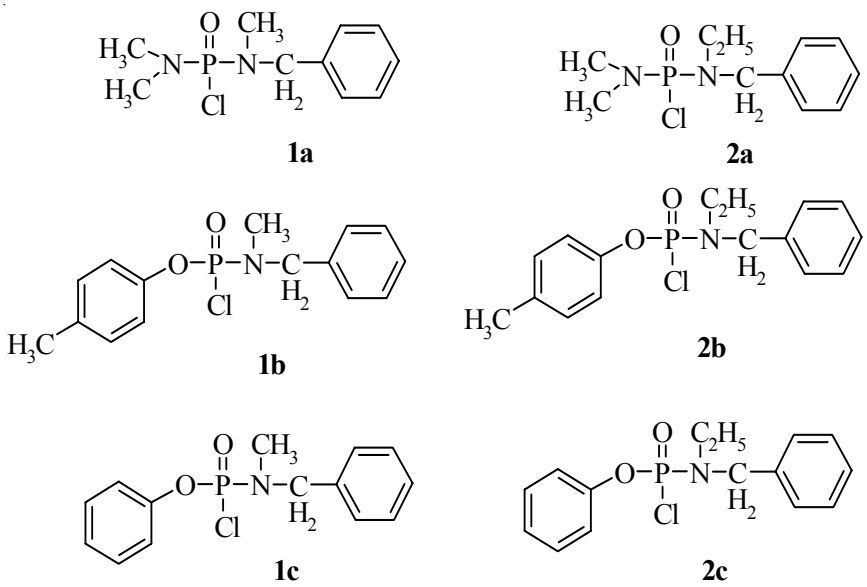
The reaction mixture for determination of the biomolecular rate constant (k_i) was: DTNB, ASCh; solution of the enzyme and phosphate buffer (same as above) plus varied concentration of inhibitors (**1a, 2a, 1b, 2b, 1c** and **2c**). The rate of hydrolysis of acetylthiocholine was monitored at 412 nm at 1 min interval for 20 min after the addition of enzyme. The biomolecular rate constant (k_i) were determined in triplicate by plotting the slopes against concentration of inhibitors and resulting slope was analyzed by linear regression.

A test tube containing all of above materials except the inhibitor was used to determine the activity of enzyme.

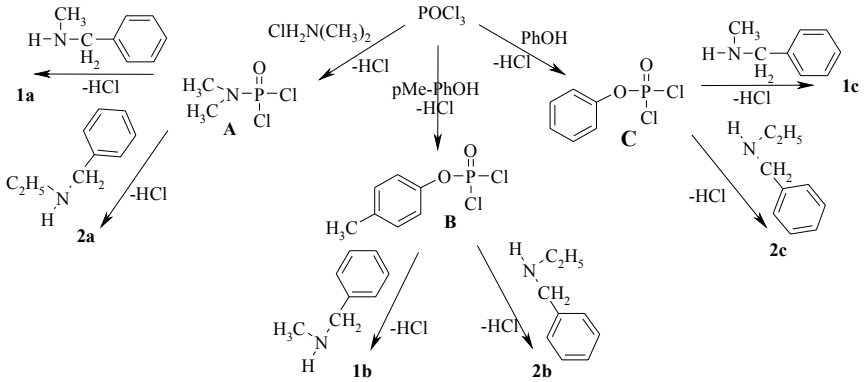
Measurement of hydrophobicity: Hydrophobic parameter of compounds was determined by measurement of octanol-water partition coefficient by shake-flask technique[13]. The aqueous phase stock solution were shaken with excess of octanol to presaturate them and were allowed to stand overnight before use. 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. The three concentrations (0.01, 0.05, 0.001 M) of solutes were prepared in octanol and then 2.5 mL of these solutions was added to 500 mL water and shaken well. The reaction solution was allowed to stand for 0.5 h, 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.

RESULTS AND DISCUSSION

As indicate in **Scheme-I**, we synthesized a series of phosphoramidates with various substituents based on phosphoryl chloride as a common starting material. Preparation of these compounds involved two subsequent nucleophilic displacements at the phosphoryl center (**Scheme-II**).



Scheme-I



Scheme-II

The structure assignment of all the isolated products can be readily deduced from their ^{1}H, ^{31}P and ^{13}C NMR spectroscopy. A summary of the NMR parameters of these compounds are given in Table-1. It is obvious that various parameters affect on ^{31}P chemical shifts, such as electronegativity of substituents and bond angle[14]. Much attention has been given to the variation of the ^{31}P chemical shifts when changes in the electronegativity of attached groups are made, the more electronegative groups cause deshilding of the phosphorus atom. Herein, it seems above factor affect on the chemical shift of phosphorus. The ^{31}P chemical shifts of synthesized compounds migrate to lower field as the electronegativity of the substituents increase. ^{31}P NMR spectra indicated that amount of deshilding in selected compounds was: **1c** > **2c** > **1b** > **2b** > **1a** > **2a**. The ^{1}H NMR spectra of compounds **1b** and **2b** showed long-range coupling between phosphorus and terminal methyl of *p*-cresol with $^{7}J_{PH}$ = 2.20 and 2.34, respectively. Similar phenomena have also been observed in some organophosphorus compounds[10].

The main absorption bands in the IR spectra of the synthesized compounds, together with their assignments are given in Table-1. The stretching vibrations of ν(P=O) appear in the range 1279-1232 cm^{-1}. The medium absorption bands observed from 954 to 902 cm^{-1} belongs to the ν(P-N) stretching vibrations of the amine nitrogen.

The elemental analysis confirmed ^{1}H, ^{13}C, ^{31}P NMR and IR spectroscopy data for the synthesized compounds.

Enzymatic evaluation: Enzymatic experiments on compounds **1a, 2a, 1b, 2b, 1c** and **2c** were performed to illustrate the inhibition behaviour and different biological activity characteristics of these 6 organophosphorus compounds. The plot of V_i/V_o against log [I], where [I] is the inhibitor concentration, gave the IC_{50} values of compounds **1a, 2a, 1b, 2b, 1c** and **2c** were 2.33×10^{-1}, 1.58×10^{-1}, 1.40×10^{-2}, 7.9×10^{-3}, 3.16×10^{-3}, 1.99×10^{-3} mM, respectively (Table-1).

TABLE-1
SPECTRAL AND ENZYMATIC DATA FOR
COMPOUNDS **1a**, **2a**, **1b**, **2b**, **1c** AND **2c**

Compounds	δP (ppm)	ν(P=O) cm^{-1}	ν(P–Cl) cm^{-1}	log P	IC_{50} (mM)	K_i (M^{-1} min^{-1})
$(CH_3)_2NPOClN(CH_2C_6H_5)(CH_3)$ (**1a**)	9.66	1239	594	2.02	2.33×10^{-1}	2.40×10^{-3}
$(CH_3)_2NPOClN(CH_2C_6H_5)(C_2H_5)$ (**2a**)	9.45	1242	596	2.37	1.58×10^{-1}	5.20×10^{-3}
$(CH_3C_6H_4O)POClN(CH_2C_6H_5)(CH_3)$ (**1b**)	12.88	1272	601	4.36	1.40×10^{-2}	1.59×10^{-2}
$(CH_3C_6H_4O)POClN(CH_2C_6H_5)(C_2H_5)$ (**2b**)	12.11	1278	603	4.95	7.90×10^{-3}	8.48×10^{-2}
$(C_6H_5O)POClN(CH_2C_6H_5)(CH_3)$ (1c)	13.93	1269	600	3.81	3.16×10^{-3}	2.16×10^{-1}
$(C_6H_5O)POClN(CH_2C_6H_5)(C_2H_5)$ (**1c**)	13.74	1279	601	4.24	1.99×10^{-3}	5.23×10^{-1}

As shown in Fig. 1 the least squares linear regression of ln of the fraction of remaining AChE activity *vs.* 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 *vs*. k_{app} resulted in a line with slope = $1/k_i$. The k_i values of the selected compounds varied from 5.23×10^{-1} to 4.8×10^{-3} M^{-1} min^{-1} (Table-1). log P were determined to illustrate the hydrophobicity of selected inhibitors **1a, 2a, 1b, 2b, 1c** and **2c** and it were 2.02, 2.37, 4.36, 4.95, 3.81 and 4.24, respectively (Table-1).

The purpose of this study to compare the *in vitro* sensitivities of hAChE inhibition by 6 phosphoramidate compounds. Two different approaches were used to assess hAChE sensitivity to inhibition by the phosphoramidate compounds[15,16]. In the first, the concentration of inhibitor required to cause 50 % inhibition (IC_{50}) of hAChE. The second approach required determination of the pseudo first-order rate constant (k_i) for enzyme inhibition. The k_i is a measure of inhibitory power the phosphoramidate compounds.

The inhibition potency of the synthesized compounds indicates that an increasing of the inhibitory effect on the hAChE is as follow: **2c > 1c > 2b > 1b > 2a > 1a**. This order was obtained by the comparison of IC_{50} and k_i values.

The IC_{50} values of the tested compounds varied between 2.33×10^{-1} and 1.99×10^{-3} mM (Table-1). These results are in accordance with those previously reported values for other phosphoramidate compounds[8]. Biomoulecular rate constants (k_i) for inhibition of hAChE were between 2.4×10^{-3} and 5.23×10^{-1} M^{-1} min^{-1}.

The inhibitory potency of organophosphorus compounds is dependent upon the leaving group, charge on phosphorus atom, hydrophobicity, stereochemistry and phosphorus atom substitutes. This behaviour is determined with respect to their structural features as well as hydrophobicity, electronic and steric parameters[17-22].

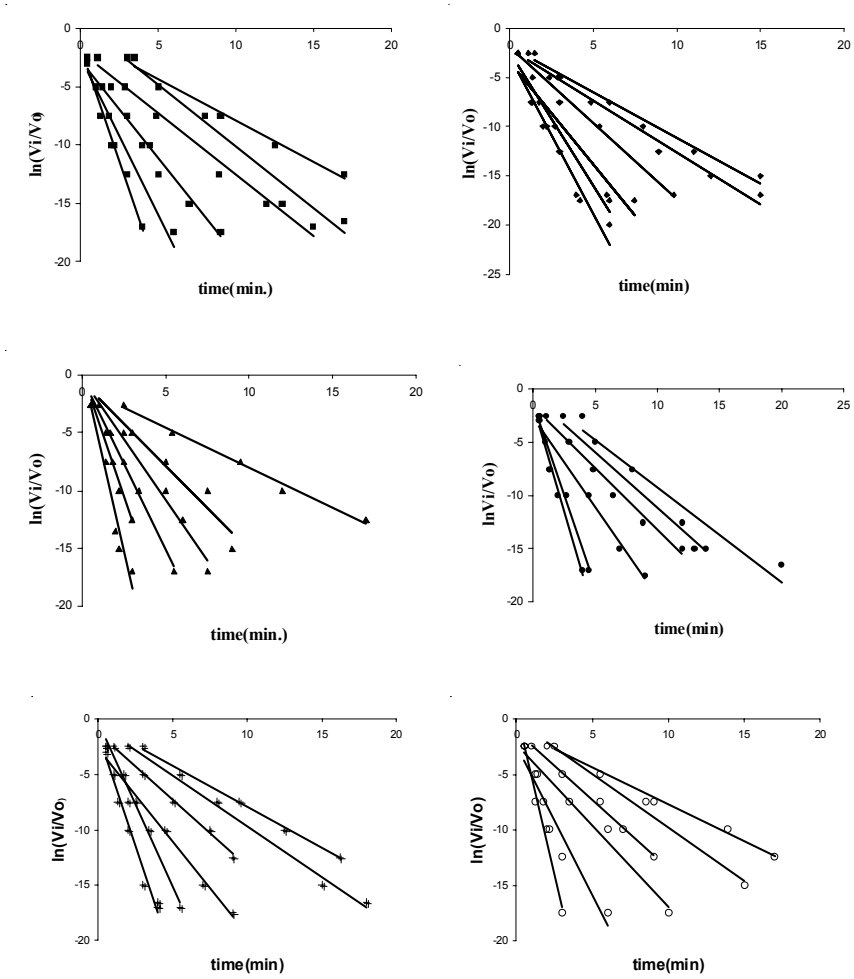


Fig. 1. Inhibitation of human erythrocyte AChE by selected phosphoramidate compounds at different concentration. Inhibitor concentration (mM) were, **1a**: 0.010253, 0.0061, 0.0047, 0.00295, 0.0025, 0.00205; **2a**: 0.015335, 0.013, 0.01, 0.008, 0.006; **1b**: 0.064, 0.117785, 0.045, 0.035, 0.02, 0.0125; **2b**: 0.28, 0.343, 0.16, 0.116, 0.08, 0.07; **1c**: 0.85, 0.65, 0.35, 0.3, 0.175, 0.15 and **2c**: 2.951, 1.4, 0.8, 0.7, 0.6, 0.2

Certain organophosphorus inactivates AChE by reaction at a nucleophilic serine hydroxyl to form a phosphoserine linkage, concomitant with the ejection of the living group[18,22]. In all the synthesized compounds, with attention bonding energy in IR spectra, chlorine atom of P-Cl bond is the best leaving group in accord with similar compound. $(EtO)_2P(O)Cl$ is a known AChE inhibitor with chloride as the suggested leaving group[16]. Hence, inhibition of hAChE by selected compounds probably was resulted

from lability of P-Cl bond that phosphorylate the active site of hAChE by phosphoryl residue. This mater is confirmed by the thermochemical bond energies in phosphorus compounds[14]. As shown in Table-1, ν(P-Cl) for all the compounds is very close to each other. Therefore different inhibitory potency of these compounds can not be relevant to leaving group.

There is considerable evidence from QSAR studies that hydrophobicity plays an important role in the action of a wide variety of inhibitors of vertebrates cholinesterase and the decrease in hydrophobicity value tends to increase with IC_{50}[13,17,23,24]. The octanol/water partition coefficient (log p) is the standard quantity to characterize the hydrophobicity/hydrophilicity of a molecule[19]. In the synthesized compounds, replacement of methyl group by ethyl group increase hydrophobicity and also, the hydrophobicity decrease when phenolic groups were replaced by amine groups and log p values varied from 2.02 to 4.95.

Electrophilicity of phosphorus atom increases its reactivity toward nucleophilic such as water or active site of serine of hAChE, is also the reason of different inhibitory for selected compounds[25,26]. ^{31}P NMR spectra for these compounds showed that phosphorus chemical shift of compounds varied from 9.45 to 13.93 ppm (chemical shifts are **1c** > **2c** > **1b** > **2b** > **1a** > **2a**). The **1c** and **2c** have the larger amount of chemical shift than **1a, 2a, 1b** and **2b**. The IC_{50} and k_i values also indicate that these compounds have the largest affinity toward the active site of hAChE. An explanation for the greater inhibition of hAChE by the **1c** and **2c** perhaps is more electronegativy of phosphorus substituents, which results in correspondingly greater reduction in electron density around phosphorus atom. This effect enhances the electrophilicity of phosphorus and thereby increases its inhibitory potency. When the phenol group in **1c** and **2c** replaced by Me_2N as an electron donor group in the **1a** and **2a**, inhibitory of them drastically decreased. The greater inhibitory potency of **1c** and **2c** relative to **1b** and **2b** is perhaps due to the low electron density of phenolic group in **1c** and **2c** than *p*-cresol group in **1b** and **2b**.

Surprisingly, the compounds with $R_2 = N(CH_3)(CH_2C_6H_5)$, (**1a**, **1b** and **1c**), which have a lower amount of phosphorus chemical shifts are the more potent inhibitor than the compounds with $R_2 = N(C_2H_5)(CH_2C_6H_5)$, (**2a**, **2b** and **2c**). This mater was perhaps due to hydrophobicity of phosphorus substituents.

It is known that, substitution of ester moiety (-OR) with amino group ($-NR_2$) in organophosphorus compounds decreased anticholinestrases activity[8,27]. Herein replacement of amino group of compounds **1a** and **2a** by *p*-cresol (in **1b** and **2b**) or phenol (in **1c** and **2c**) must increase activity toward the active site of hAChE. This was confirmed by the comparison of IC_{50} value of selected compound. IC_{50} for compounds $(C_4H_9O)(C_4H_9NH)P(O)$

(*o*-2,5-dichlorophenyl) and $(C_4H_9NH)_2P(O)$(*o*-2,5-dichlorophenyl) obtained 2.5×10^{-6} and 7.3×10^{-5} M for hAChE, respectively, which is comparable to previous results[8].

In summary, some phosphoramidate compounds **1a**, **2a**, **1c** and **2c** were synthesized and characterized. The kinetic parameters (IC_{50} and k_i) and hydrophobicity of **1a**, **2a**, **1b**, **2b**, **1c** and **2c** were determined. The present analysis of the factors that influence the difference in reactivity of selected inhibitors of hAChE indicated that the hydrophobicity may be as important as their electronic effect determining their effectiveness as AChE inhibitors. The inhibitory potency of the synthesized compounds decreased from following order: **2c** > **1c** > **2b** > **1b** > **2a** > **1a**. The Michaelis-Menten plots indicate an irreversible inhibitory for compounds **1a, 2a, 1b, 2b, 1c** and **2c**.

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