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INHIBITION OF ACETYLCHOLINESTERASE BY THREE NEW PYRIDINIUM COMPOUNDS AND THEIR EFFECT ON PHOSPHONYLATION OF THE ENZYME

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Three new mono-pyridinium compounds were prepared: 1-phenacyl-2-methylpyridinium chloride (**1**), 1-benzoylethylpyridinium chloride (**2**) and 1-benzoylethylpyridinium-4-aldoxime chloride (**3**) and assayed *in vitro* for their inhibitory effect on human blood acetylcholinesterase (EC 3.1.1.7, AChE). All the three compounds inhibited AChE reversibly; their binding affinity for the enzyme was compared with their protective effect (PI) on AChE phosphonylation by soman and VX. Compound **1** was found to bind to both the catalytic and the allosteric (substrate inhibition) sites of the enzyme with estimated dissociation constants of 6.9 μM (K_{cat}) and 27 μM (K_{all}), respectively. Compound **2** bound to the catalytic site with $K_{cat} = 59\,\mu M$ and compound **3** only to the allosteric site with $K_{all} = 328\,\mu M$. PI was evaluated from phosphonylation measured in the absence and in presence of the compounds applied in a concentration corresponding to their K_{cat} or K_{all} value, and was also calculated from theoretical equations deduced from the reversible inhibition of the enzyme. Compounds **1** and **3** protected the enzyme from phosphonylation by soman and VX, whereas no protection was observed in the presence of compound **2** under the same conditions. Irrespective of the binding sites to AChE, PI for compounds **1** and **3** evaluated from phosphonylation agreed with PI calculated from reversible inhibition. Compound **3** was found to be a weak reactivator of methylphosphonylated AChE with $k_r = 1.1 \times 10^2\,L\,mol^{-1}\,min^{-1}$.

Keywords: Mono-pyridinium compounds; Acetylcholinesterase; Reversible inhibition; Soman; VX; Protection; Reactivation

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INTRODUCTION

Acetylcholinesterase (EC 3.1.1.7, AChE) is a serine hydrolase present at all cholinergic synapses, with the physiological role of hydrolysing the neurotransmitter acetylcholine (AChE).[1] This enzyme is the major target in poisoning by organophosphorus compounds (OPs) which have wide agricultural, medical and military application. OPs inhibit AChE by rapid phosphylation (phosphorylation or phosphonylation) of its active-site serine.[2,3] Phosphylated AChE can be reactivated by nucleophilic agents of which the most efficient in present conventional medical treatment are pyridinium oximes.[4] Their effectiveness is primarily attributed to the nucleophilic displacement of the OP moiety from the phosphylated serine.[2] Pyridinium compounds (with or without the oxime group) are reversible inhibitors of AChE; they bind to either the catalytic or the allosteric (substrate inhibition) enzyme binding site, or both, and their antidotal effect therefore reveals also protection of AChE in phosphylation.[5] The structure–function relationships among pyridinium oxime reactivators are still not clearly understood.[6] The antidotal efficiency of pyridinium compounds depends on the types of all involved components: the enzyme, the OP and the antidote itself. This limits the activity profile of commonly used reactivators and protectors, and none of the known pyridinium antidotes can therefore be regarded as a broad spectrum antidote.[4] The search for a more universal antidote is continuing, the more so since antidotal effects of some pyridinium compounds indicate other mechanisms which cannot be attributed to AChE protection or reactivation.[4,7]

The three new mono-pyridinium compounds described in this paper (Figure 1) were synthesised for further studies of the reactions with the aquapentacyanoferrate(II)ion.[8,9] According to their chemical structure they

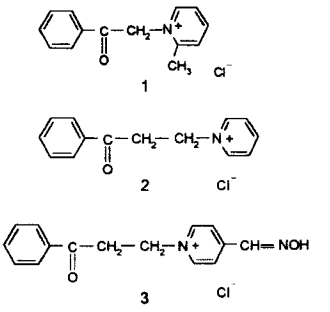

FIGURE 1 Structures of the synthesised compounds.

are cholinesterase inhibitors, and therefore possible protectors of AChE in phosphylation. This paper describes their synthesis, evaluates their reversible binding to AChE and their effect on enzyme phosphylation by soman and VX. One of the compounds bears an oxime group and was tested for its reactivating efficiency of VX-phosphylated AChE.

MATERIALS AND METHODS

Synthesis of Compounds

3-Chloropropiophenone and 2-chloroacetophenone of 98% and 99% purity were purchased from Aldrich® (Milwaukee, WI, USA). Pyridine-4-aldoxime was a Fluka Chemie AG (Buchs, Switzerland) reagent, further purified by recrystallisation from water (m.p. 130°C). 2-Methylpyridine and pyridine were of analytical reagent grade, purchased from Kemika (Zagreb, Croatia). Melting points were determined on a Culatti apparatus. IR spectra were recorded in KBr pellets on a 580-B Perkin Elmer spectrophotometer. UV spectral measurements of aqueous solutions were made at 25°C with a Hewlett Packard 8451A diode Array spectrophotometer in 1.0-cm silica–glass cells. The course of reactions was monitored and the homogeneity of products checked by TLC on Kieselgel 60 F_{254} (Merck Biochemica, Darmstadt, Germany) using BuOH–CH_3COOH–H_2O (4 : 1 : 1, v/v/v) as solvent.

1-Phenacyl-2-methylpyridinium chloride (1)

0.90 g (0.01 mol) of 2-methylpyridine in 1.7 mL acetone was added to 1.12 g of chloroacetophenone (0.072 mol) dissolved in 7 mL of acetone and the mixture was left at room temperature for 15 days. Ether (100 mL) was then added to the reaction mixture, inducing the formation of a white solid precipitate, which was filtered out, dissolved in ethanol and again precipitated by ether addition. The yield was 1.8 g (90%) of white crystals m.p. 193°C. $R_f = 0.037$. The IR spectrum showed the absorption characteristics of the carbonyl group (1710 cm^{-1}), the substitution on the aromatic rings in position 1 (698–768 cm^{-1}) and the substitution of the pyridine ring in position 2 (723 cm^{-1}). UV: $\lambda_{max} = 256$ nm, $\varepsilon = 18200\ M^{-1}\ cm^{-1}$. Found: C, 67.7; H, 5.9; N, 6.0; Cl, 13.9%. Calculated for $C_{14}H_{14}NOCl$ (M_r 247.72): C, 67.9; H, 5.7; N, 5.7; Cl, 14.3%.

1-Benzoylethylpyridinium chloride (2)

1.68 g (0.01 mol) of chloropropiophenone and 0.79 g (0.01 mol) of pyridine were dissolved in 15 mL ethanol/ether (1 : 1, v/v) and the mixture was left at

room temperature for 15 days. Ether (100 mL) was added to the reaction mixture and the obtained white precipitate was filtered out. Recrystallisation from boiling ethanol yielded 2.3 g (93% yield) of white crystals m.p. 188°C. $R_f = 0.022$. The IR spectrum showed the absorption characteristics of the carbonyl group (1690 cm^{-1}) and of the mono-substituted aromatic rings (690–770 cm^{-1}). UV: $\lambda_{max} = 252$, $\varepsilon = 14790\ M^{-1}\ cm^{-1}$. Found: C, 66.8; H, 5.4; N, 5.9; Cl, 14.0%. Calculated for $C_{14}H_{14}NOCl$ (M_r 247.73): C, 67.1; H, 5.7; N, 5.7; Cl, 14.3%.

***1-Benzoylethylpyridinium-4-aldoxime chloride* (3)**

1.68 g (0.01 mol) of chloropropiophenone and 1.22 g (0.01 mol) of pyridine-4-aldoxime were dissolved in ethanol (5 mL) before addition of ether (15 mL). The mixture was then left at room temperature for 15 days and finally evaporated to dryness. The residual pellet was dissolved in boiling ethanol and precipitated by ether. The repeated procedure yielded 2.5 g (86%) of white crystals m.p. 218°C. $R_f = 0.018$. IR spectrum showed the absorption due to the carbonyl group (1700 cm^{-1}), the oxime group (1010, 1460, 1620, 1660 and about 3000 cm^{-1}) and the substitution of the aromatic rings in positions 1 (703–768 cm^{-1}) and 4 (826 cm^{-1}). UV: $\lambda_{max} = 280$, $\varepsilon = 19050\ M^{-1}\ cm^{-1}$ and at $\lambda_{max} = 254$, $\varepsilon = 18200\ M^{-1}\ cm^{-1}$. Found: N, 9.2; Cl, 11.7%. Calculated for $C_{15}H_{15}N_2O_2Cl$ (M_r 290.75): N, 9.6; Cl, 12.2%.

Enzyme Assays

Acetylcholinesterase (EC 3.1.1.7, AChE) was obtained from native human erythrocytes of heparinized venous blood, washed and resuspended to the normal blood volume with saline. The enzyme preparation was diluted 400-fold for activity measurements. All experiments were carried out in 0.1 M phosphate buffer pH 7.4 at 37°C using acetylthiocholine iodide (ATCh) as substrate. The estimated specific activity of the enzyme preparation at 1.0 mM substrate concentration was 5.3 $\mu mol\ min^{-1}\ mL^{-1}$. The enzyme activities were measured spectrophotometrically according to the Ellman procedure[10] with the thiol reagent DTNB (5,5′-dithiobis-2-nitrobenzoic acid, 0.33 mM final concentration). Measurements were carried out against blanks of suitable composition. A more detailed description of all applied experimental procedures is given in earlier publications.[11–14]

Reversible Inhibition

Concentration ranges of pyridinium compounds and ATCh are shown in Table I. Three to five different substrate concentrations were used for each

compound. One to four different concentrations of the compound were applied at a given substrate concentration in order to obtain AChE inhibition between 20% and 80%. The increase in absorbance was measured over a period of 1 min after adding the substrate. At 1.0 mM ATCh, compound **3** slightly induced substrate hydrolysis and the measured enzyme activities were subsequently corrected. It was noted that at substrate concentrations higher than 1.0 mM in the presence of compounds **2** or **3** yet another reaction took place, which affected the amount of the yellow anion spectrophotometrically determined during the reaction. Under the experimental conditions applied in the study, this reaction did not interfere with enzyme activity measurements and was not studied any further.

Progressive Inhibition and Protection

The organophosphorus (OP) inhibitors used were soman (O-1,2,2-trimethylpropyl methylphosphonofluoridate) and VX (O-ethyl S-2-diisopropylaminoethyl methylphosphonothioate). The OP compound was added to the reaction medium containing the erythrocyte/buffer suspension, DTNB and water. Final concentrations of soman and VX in activity measurements were 5 and 20 nM respectively. After a preselected inhibition time (up to 2 min), the substrate was added (1.0 mM final concentration) and the enzyme activity determined. In measurements of the protection effect the experimental procedure was the same, except that the reaction medium contained the pyridinium compound instead of water. Final concentrations of pyridinium compounds were taken close to the values of their enzyme/compound dissociation constants for binding to the enzyme (Table I).

Reactivation

Reactivating potency was tested with the oxime compound **3** on AChE inhibited by VX. The undiluted erythrocyte preparation was incubated with 50 nM VX for 5 min (achieving 90% inhibition) and at the end of incubation diluted 400-times with 0.1 M phosphate buffer of pH 7.4 containing the oxime to start the reactivation. The final oxime concentration ranged from 0.1 to 0.4 mM. During time intervals up to 90 min (of reactivation time) aliquots were withdrawn, DTNB and ATCh (1.0 mM final concentration) added and the enzyme activity measured. Under these conditions the enzyme activity in the absence of the oxime was stable and no spontaneous reactivation of the phosphonylated enzyme took place.[12]

RESULTS AND DISCUSSION

Reversible Inhibition of AChE by Pyridinium Compounds

All three tested compounds were reversible inhibitors of AChE. The inhibition constants were estimated from the effect of substrate concentration on the degree of inhibition according to the equation:

$$K_{app} = v \cdot i/(v_0 - v) = K_{(I)} + [K_{(I)}/K_{(S)}]S \quad (1)$$

where K_{app} is the apparent enzyme/inhibitor dissociation constant at a given substrate concentration S, calculated from enzyme activities v_0 and v measured in the absence and in presence of the reversible inhibitor concentration i, respectively. $K_{(I)}$ is the dissociation constant of the enzyme/inhibitor complex formed in the catalytic (K_{cat}) or the allosteric (K_{all}) binding site.[2,15,16] $K_{(S)}$ should correspond to the Michaelis constant K_m when the inhibitor binds to the catalytic site, or to the substrate inhibition constant K_{ss} when the inhibitor binds to the allosteric (substrate inhibition) site.[2,5]

The values of $K_{(I)}$ and $K_{(S)}$ were estimated from the Hunter–Downs plot (K_{app} vs S)[17] as intercepts of the line on the ordinate and abscissa, respectively. When the reversible inhibitor binds to both, the catalytic and the allosteric binding site of AChE, K_{app} is not a linear function of S and the intercepts on the abscissa and on the ordinate are functions of both, K_m and K_{ss}, and of both AChE/inhibitor dissociation constants (K_{cat} and K_{all}), respectively.[5] K_{cat} is then obtained from the initial linear part of the curve K_{app} vs S at low ATCh concentrations (up to 1.0 mM) at which inhibitor competes for the binding to the catalytic site, and K_{all} from the linear part of the curve at high ATCh concentrations (above 1.0 mM) at which inhibitor competes for binding to the allosteric site.

The estimated kinetic constants for the binding to the catalytic and/or allosteric site are given in Table I. The catalytic site of AChE shows the highest affinity for compound **1** and the lowest for compound **3**. The AChE inhibition by compound **1** was determined at 0.1–1.0 mM ATCh and by compound **2** at 0.1–0.5 mM ATCh. The value of K_{cat} obtained for compound **1** is, to the best of our knowledge, the lowest value obtained for mono- and bis-pyridinium compounds[5,13] and indicates a strong affinity of AChE for this compound. The affinity for compound **2** was lower and similar to affinities reported for other pyridinium compounds.

The allosteric binding of compound **1** was measured at 1.0–10 mM ATCh concentrations. The obtained value for K_{all} (Table I) was similar to the

TABLE I Kinetic constants for reversible binding of pyridinium compounds to AChE with ATCh as substrate

Compound	*Concentration* (mM)	*Substrate range* (mM)	K_{cat} (μM)	K_{all} (μM)	$^*K_{(S)}$ (mM)
1	0.01–0.10	0.10–1.0	6.9 ± 1.4	—	0.22 ± 0.05
	0.05–0.50	1, 5, 10	—	27 ± 10	2.3 ± 0.9
2	0.10–0.50	0.10–0.50	59 ± 4.3	—	0.26 ± 0.02
3	0.25, 0.50	0.10–1.0	—	328 ± 48	—

K_{cat} and K_{all} are AChE/inhibitor dissociation constants for the catalytic and allosteric binding site respectively, and $K_{(S)}$ is the dissociation constant of the AChE/substrate complex formed in the catalytic or the allosteric binding site. The constants were evaluated from the Hunter–Downs plot as described in the text and expressed as mean ± SE from 4 to 8 different combinations of the substrate/compound concentrations.
*Literature data for K_m: 0.09–0.14 mM and K_{ss}: 3.3–29 mM.

values for the two imidazolium oximes BDB-106 and BDB-108, which bind only to the allosteric AChE site,[18] and about 100 times lower than K_{all} reported for 4,4′-bipyridine[19] which binds to both binding sites. The indication of a potential allosteric binding of compound **2** to AChE could not be checked further because of the experimental limitations explained above.

The $K_{(S)}$ values for compounds **1** and **2** (Table I) were compared to published values of K_m and K_{ss} for AChE and substrate ATCh.[2,10,20,21] The values of $K_{(S)}$ for compounds **1** and **2** obtained at low ATCh concentrations were somewhat higher than those published for K_m, and the $K_{(S)}$ value for compound **1** obtained at high substrate concentrations was lower than those published for K_{ss}. It is considered (cf. Ref. [5]) that such deviation in binding of the reversible ligand to both enzyme binding sites is due to the fact that K_{app} is a non-linear function of S, and that intercepts on the ordinate and abscissa are functions of both enzyme/inhibitor dissociation constants, K_{cat} and K_{all}, and of both enzyme/substrate constants, K_m and K_{ss}, respectively. Therefore, the values of $K_{(S)}$ obtained for compounds **1** and **2** (Table I) were taken to correspond to K_m or K_{ss}, respectively, and thereby support the conclusion that the compounds bind to the AChE catalytic site in addition to the enzyme allosteric site for compound **1**.

Compound **3**, to which AChE showed the lowest affinity (Table I), inhibited AChE non-competitively. The value for K_{app} was the same at 0.1 and 1.0 mM ATCh and it was therefore concluded that the compound binds only to the allosteric site. To the best of our knowledge no pyridinium compound has so far been reported to bind to AChE only at the allosteric site. In comparison to the imidazolium oximes which also bind only allosterically[18] the affinity of the enzyme for compound **3** was more than 10-times weaker.

Protective Effect of Mono-pyridinium Compounds on Phosphonylation of AChE

Pyridinium compounds exert their protective effect by binding to the anionic subsite of the AChE catalytic center so that they interfere with the binding domain of the acylating site ligands and affect their reversible association with the enzyme.[1] For ligands which bind to the AChE allosteric site, it has been shown that they decrease the association and dissociation rate of an acylation site ligand, and in this way allosterically bound pyridinium oximes also protect the enzyme from phosphylation. The actual mechanism by which allosteric ligands exert their inhibitory effect is still unclear.[22–25]

The effect of pyridinium compounds on phosphonylation of AChE was studied with soman and VX as organophosphorus inhibitors and expressed as a protective index (PI). The protective indices were evaluated both experimentally (PI_{exp}) from progressive inhibition, and theoretically (PI_{calc}) using equations based on the kinetic parameters for binding of reversible ligands to the enzyme.[5,19]

PI_{exp} was estimated from the relationship $PI = k/k'$ by determining the second-order rate constants of phosphonylation in the absence (k) and presence (k') of the pyridinium compound at a concentration corresponding to its K_{cat} and/or K_{all} value.

PI_{calc} are theoretical values expected from the binding sites of the reversible ligand to the enzyme.[19] The equation $PI = 1 + i/K$ holds for binding to one site only, either catalytic or allosteric, and K stands for K_{cat} or K_{all}. When binding occurs at both sites, PI is then a function of the two inhibition constants; if the ligand forms two binary complexes (one in catalytic and one in allosteric site) then PI is described by

$$PI = 1 + i/K_{cat} + i/K_{all} \tag{2}$$

or, when a ternary complex is formed additionally due to binding of the ligand to both sites simultaneously by

$$PI = (1 + i/K_{cat})(1 + i/K_{all}). \tag{3}$$

Estimated protective indices for tested pyridinium compounds are presented in Table II. Compounds **1** and **3** protected the enzyme from phosphonylation and irrespective of the differences in their binding to AChE the protective indices evaluated from progressive inhibition agreed with those calculated from theoretical equations. Under the same experimental conditions compound **2** had no effect on phosphonylation and we do not have a reasonable explanation for this so far.

TABLE II The protective effect of pyridinium compounds on phosphonylation of AChE by soman and VX expressed as protective index (PI)

Compound	*Concentration* (μM)	PI_{exp}		PI_{calc}		
		Soman	*VX*	*One binding site**	*Two binding sites*	
					Equation (2)	*Equation* (3)
1	7	2.2	2.2	2.0	2.3	4.2
	30	6.7	7.1	5.3	6.5	11
2	60	1.0	1.0	2.0	—	—
3	300	2.0	2.3	2.0	—	—

PI_{exp} values were obtained experimentally from the ratio of the second-order rate constants of phosphonylation with 5 nM soman or 20 nM VX in the absence and in the presence of the pyridinium compound; the values are the mean of 2–3 experiments. PI_{calc} values were calculated according to theoretical equations which correspond to binding of the reversible inhibitor in the catalytic, allosteric or in both enzyme binding sites as described in the text using values of K_{cat} and K_{all} from Table I.
*From $PI = 1 + i/K$.

For compound **1**, PI_{exp} obtained at the concentration corresponding to its K_{all} value was in accordance with PI_{calc} derived by Equation (2) which indicates that the protective effect is produced through formation of binary complexes in each of the two binding sites without formation of a ternary complex.[19] PI_{exp} obtained for compound **3** corresponded to PI_{calc} derived from $PI = 1 + i/K$, confirming that the protection with this compound results only from binding to the allosteric site as was found earlier for imidazolium oximes.[18]

Reactivation of Phosphonylated AChE

Besides its protective effect the oxime compound **3** was also shown to be a reactivator of the VX-phosphonylated AChE but of rather weak nucleophilicity. The second-order rate constant for reactivation k_r was calculated from

$$\ln[(v - v_1)/(v - v_t)] = k \cdot t = k_r \cdot [\text{oxime}] \cdot t, \quad (4)$$

where v stands for the activity of the non-phosphonylated enzyme but in the presence of the oxime, v_1 and v_t are activities of the phosphonylated enzyme after one and t minutes of reaction with the oxime and k is the first-order rate constant for reactivation. The reactivation was measured with four different oxime concentrations and the first-order rate constants k were estimated by plotting $\ln[(v - v_1)/(v - v_t)]$ against the time of reaction with the oxime. Depending on the oxime concentration, the time course for reactivation deviated from linearity after about 10 min, as also found for other pyridinium compounds.[12,14,18] The constants were therefore evaluated from

the initial linear part of the reactivation curves; the calculation was based on the total oxime concentration. Compared to the literature data for pyridinium oximes[12,14,18] compound **3** showed a very low efficiency for reactivating the ethylmethyl-phosphonylated AChE, with a second-order rate constant for reactivation $k_r = 1.1 \times 10^2\ \mathrm{L\ mol^{-1}\ min^{-1}}$.

Mono-pyridinium oximes are known to be less efficient reactivators of phosphylated AChE than bis-pyridinium oximes.[26] Binding modes of oximes to the phosphylated and non-phosphylated AChE have been shown to be different, and also their interactions with the bound phosphyl moieties can affect the reactivation process.[27] It was also found that some organophosphorus compounds in addition to binding to the catalytic site can also bind to the AChE allosteric site[2,28,29] which may affect the protective efficiency of allosterically bound pyridinium oximes. An allosteric site action of VX was, under our experimental conditions, not demonstrated.[14,19] The fact that compound **3** is bound only allosterically may also influence its suitability to undergo nucleophilic attack at the catalytic serine so lessening its reactivating efficiency.

According to literature data 1-phenacyloxime pyridinium chloride and its derivatives exerted *in vivo* a very complex action on the cholinergic and adrenergic nervous system and a strong anticholinesterase effect, but did not reactivate diethylphosphorylated cholinesterase *in vitro*.[30] The high affinity for AChE and the protection efficiency observed with compound **1**, and also the possible antidotal effects not related to AChE protection or reactivation by compounds **1** and **3**, would justify their *in vivo* testing.

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