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Quinuclidinium-imidazolium compounds: synthesis, mode of interaction with acetylcholinesterase and effect upon Soman intoxicated mice

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Abstract Four compounds were prepared: 3-oxo-1-methylquinuclidinium iodide (**I**), 2-hydroxyiminomethyl-1,3-dimethylimidazolium iodide (**II**) and two conjugates of **I** and **II** linked by -$(CH_2)_3$- (**III**) and -CH_2-O-CH_2- (**IV**). The aim was to evaluate separately the properties of **I** and **II** as opposed to **III** and **IV**, which contain both moieties in the same molecule. All four compounds were reversible inhibitors of acetylcholinesterase (AChE; EC 3.1.1.7). The enzyme/inhibitor dissociation constants for the catalytic site ranged from 0.073 mM (**II**) to 1.6 mM (**I**). The dissociation constant of **I** for the allosteric (substrate inhibition) site was 4.8 mM. Possible binding of the other compounds to the allosteric site could not be measured because **II**, **III** and **IV** reacted with the substrate acetylthiocholine (ATCh) and at high ATCh concentrations the non-enzymic reaction interfered with the enzymic hydrolysis of ATCh. The rate constants for the non-enzymic ATCh hydrolysis were between 23 and 37 l/mol per min. All four compounds protected AChE against phosphorylation by Soman and VX. The protective index (PI) of **I** (calculated from binding of **I** to both, catalytic and allosteric sites in AChE) agreed with the measured PI; this confirms that allosteric binding contributes to the decrease of phosphorylation rates. The PI values obtained with **III** and **IV** were higher than those predicted by the assumption of their binding to the AChE catalytic site only. The toxicity (i.p. LD_{50}) of compounds **I**, **II**, **III** and **IV** for mice was 0.21, 0.68, 0.49 and 0.77 mmol/kg body wt. respectively. All four compounds protected mice against Soman when given (i.p.) together with atropine 1 min after Soman (s.c.). One-quarter of the LD_{50} dose fully protected mice (survival of all animals) against 2.52 (**IV**), 2.00 (**I** and **III**) and 1.58 (**II**) LD_{50} doses of Soman.

Key words Acetylcholinesterase · Quinuclidine-imidazolium compounds · Reversible inhibition and protection of acetylcholinesterase against phosphorylation · Antidotal effect against Soman poisoned mice · Soman

Abbreviations *DTNB* 5,5′-Dithiobis (2-nitrobenzoic acid) · *Soman* O-1,2,2-trimethylpropyl methylphosphonofluoridate · *VX* O-ethyl S-2-diisopropylaminoethyl methylphosphonothioate

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Introduction

Oximes are reactivators of phosphorylated acetylcholinesterase (AChE; EC 3.1.1.7) and are therefore used in the therapy of poisoning by organophosphorus (OP) compounds. AChE has two binding sites for many reversible ligands: the catalytic site and an allosteric site. Several imidazolium and pyridinium oximes have been shown to bind reversibly to both of these sites (cf. Reiner et al. 1996). The substrates, acetylcholine and acetylthiocholine also bind to both sites; binding of substrates to the allosteric site causes substrate inhibition (cf. Aldridge and Reiner 1972; Radić et al. 1991).

Reversible ligands protect the catalytic site against phosphorylation due to a direct competition between the ligand and the OP compound. Protection of the catalytic site may, however, also be achieved with ligands which bind to the allosteric site only. This was proved for the allosteric ligands, propidium and one coumarin derivative (Aldridge and Reiner 1969, 1972; Radić et al. 1984, 1991; Taylor and Lappi 1975), and suggested for two bis-imidazolium oximes (Francišković et al. 1993).

Four compounds are described in this paper (Table 1): a 3-oxoquinuclidinium derivative (**I**), an imidazolium oxime (**II**) and two conjugates of 3-oxoquinuclidinium and the imidazolium oxime (**III** and **IV**). Compound **I** was shown by Sterling et al. (1988) to protect rats against the toxicity of Soman, when administered in conjunction with atropine and N-methyl-2-hydroxyiminomethylpyridinium chloride (2-PAM). Compound **II** was shown by Galoši et al. (1988) to be an inhibitor of AChE and reactivator of Sarin-phosphorylated AChE. It was further shown that some conjugates of 3-substituted quinuclidinium derivatives with pyridinium oximes, prepared by Amitai et al. (1987), were antidotes against Tabun and Soman poisoning in dogs and monkeys (Amitai et al. 1995).

In the present paper, conjugates of the 3-oxoquinuclidinium derivative (**I**) with an imidazolium oxime (**II**) were prepared and studied for their mode of binding to AChE, their effect upon phosphorylation of AChE by Soman and VX, and their effect upon Soman intoxicated mice. These compounds (**III** and **IV**) have not been previously described. The aim was to evaluate separately the properties of compounds **I** and **II**, as opposed to compounds **III** and **IV**, which contain both moieties in the same molecule. None of the four compounds has been investigated to date for their mode of interaction with AChE.

Materials and methods

Synthesis of compounds

Formylation of 1-methylimidazole was performed by the method of Iversen and Lund (1966). The aldehyde was converted to the oxime derivative in a standard fashion. 3-Oxo-1-(chloromethoxymethyl)quinuclidinium chloride (1), 3-oxo-1-(3-iodopropyl)quinuclidinium iodide (2) and 3-oxo-1-(3-bromopropyl)quinuclidinium bromide (3) were prepared by a modified method of Amitai et al. (1987). 3-Oxo-l-methylquinuclidinium iodide (compound **I**) and 2-hydroxyiminomethyl-1,3-dimethylimidazolium iodide (compound **II**) were synthesized according to the procedure described by Galoši et al. (1988) and Grifantini et al. (1972).

3-Oxo-1-[3-(2-hydroxyiminomethyl-3-methyl-l-imidazolio)propyl]-quinuclidinium diiodide (compound **IIIa**) and dibromide (compound **IIIb**), and 3-oxo-1-[3-(2-hydroxyiminomethyl-3-methyl -l-imidazolio)-2-oxapropyl]quinuclidinium dichloride (compound **IV**) were synthesized by the reaction of (1), (2) or (3) with one equivalent of 1-methylimidazole-2-aldoxime in dry dimethyl formamide DMF at room temperature for 24 h. The crystals were separated by filtration and washed with ether. Analytical and spectral data of compounds **I**–**IV** are given in Table 1. Compound **III** was prepared as an iodide (**a**) and bromide (**b**). Studies in vitro were done with **IIIa** and in vivo with **IIIb**.

Studies in vitro

The source of AChE was native human erythrocytes; the final dilution during enzyme assay was 400-fold. The substrate was acetylthiocholine iodide (ATCh). All experiments were done in 0.1 M phosphate buffer, pH 7.4, at 37 °C. The enzyme activity was measured by the spectrophotometric method of Ellman et al. (1961) with the thiol reagent DTNB (final conc. 0.33 mM). The reaction was performed in a total volume of 3.0 ml. The increase in absorbance was read at 412 nm, at 15 s intervals, against a blank which contained the erythrocytes suspended in buffer and DTNB. Enzyme activities were corrected for substrate hydrolysis due to the reaction of acetylthiocholine with the oxime. At 5 and 10 mM substrate, activities were also corrected for spontaneous substrate hydrolysis.

The reaction of acetylthiocholine with the oxime (compounds **II**, **III** and **IV**) was measured in a medium which contained 0.1 M phosphate buffer, DTNB, substrate and oxime. The increase in absorbance was read for 1 min against a blank which contained buffer and DTNB. The oximes did not react with DTNB and blank samples therefore did not contain the oximes. Concentrations of the oximes and of the substrate are given in Table 2. Two to four measurements were done with each substrate/oxime concentration pair. The spontaneous substrate hydrolysis was measured in a medium which contained buffer, DTNB and substrate; the blank contained buffer and DTNB.

Reversible inhibition of AChE was measured in a medium which contained the erythrocytes suspended in buffer, DTNB, the studied compound and the substrate. Control samples contained no studied compound. The increase in absorbance was read for 1 min. The concentrations of the studied compounds and of the substrate are given in Table 3. Two to four measurements were done with each substrate/compound concentration pair. Protection of AChE against phosphorylation was measured in a medium which contained the erythrocytes suspended in buffer, DTNB, the studied compound and the OP compound. After a given time of inhibition (up to 4 min) the substrate was added (final concentration 1.0 mM) and the increase in absorbance read for 1 min. Control samples contained no studied compound.

Studies in vivo

Male BALB-C mice (18–25 g body wt.) were fed on a standard diet, which was withdrawn 24 h prior to treatment. Animals had free access to water and were kept in a room maintained by thermostat at 21 °C in Macralone cages under a light/dark cycle of 12:12 h. The acute i.p. toxicity (LD_{50}) was based upon 24 h mortality rates calculated according to Thompson (1947) and Weil (1952). Each LD_{50} was evaluated from results obtained with 4–6 doses of a given compound (dissolved in water); six animals were injected per dose; whenever the results of the experiment allowed, the 95% confidence limits were estimated from the tables (Thompson 1947; Weil 1952).

The therapeutic effect against Soman intoxication was tested by giving the studied compounds (1/4 of their LD_{50} dose) together with atropine sulphate (10 mg/kg), or atropine sulphate only, immediately after Soman. Compounds **I**–**IV** and atropine sulphate were given i.p.; Soman was given s.c. The efficacy of compounds was expressed as therapeutic factor (TF) and therapeutic dose (TD). The therapeutic factor was calculated from:(LD_{50} of Soman plus antidote)/(LD_{50} of Soman only). The therapeutic dose is the highest multiple of the LD_{50} of Soman, which was fully counteracted (survival of all animals) by the antidotes.

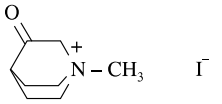
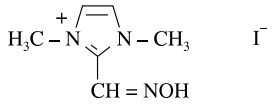
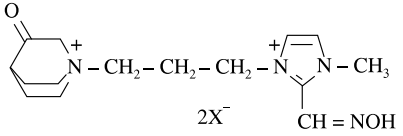
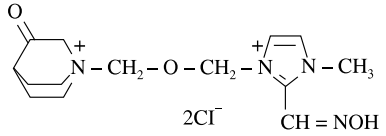
Results and discussion

Reaction of acetylthiocholine with the imidazolium oximes

It has been shown earlier that oximes react with ATCh whereby thiocholine is one of the reaction products (cf. Škrinjarić-Špoljar et al. 1992). It has further been shown that the reaction occurs on a 1:1 molar basis (Škrinjarić-Špoljar et al. 1992).

All three studied oximes reacted with ATCh. The second-order rate constant of the reaction (k) was calculated according to:

Table 1 Structure and physical data of prepared compounds (*NMR* Nuclear magnetic resonance, *DMSO* dimethyl sulphoxide)

Compound	Structure	m.p.[a] (°C)	Yield (%)	IR[b] (cm^{-1})	^{1}H NMR[c] (ppm)	^{13}C NMR[c] (ppm)
I		278	97.5	2923, 1753, 1468, 1120, 1090, 945, 786, 479	2.01–2.17 (m, 2H); 2.17–2.35 (m, 2H); 2.70–2.74 (m, 1H); 3.16 (s, 3H); 3.55–3.67 (m, 2H); 3.67–3.80 (m, 2H); 4.33 (s, 2H)	20.87, 36.54, 52.55, 56.03, 67.10, 203.43
II		213–215	92.2	3040–3240, 990, 1530	3.9 (s, 6H); 7.8 (s, 2H); 8.5 (s, 1H); 12.95 (s, 1H)	36.80, 24.44, 135.87, 137.26
III		**a** (X = I) 217–219	35.0	3500–2820, 3120, 3040, 1747, 1645, 1530, 1408, 1242, 990	2.00–2.19 (m, 2H); 2.20–2.35 (m, 4H); 2.70–2.76 (m, 1H); 3.40–3.50 (m, 2H); 3.51–3.65 (m, 2H); 3.96 (s, 3H); 4.25 (s, 2H); 4.40 (t, 2H, J = 7 Hz); 7.91 (s, 2H); 8.62 (s, 1H); 13.05 (s, 1H)	20.67, 22.55, 36.68, 37.10, 45.80, 54.47, 60.08, 65.07, 123.25, 125.27, 135.81, 137.24, 203.05
		b (X = Br) 215–218	32.5	2500–3150, 1751, 1522, 988	[d]	[d]
IV		174.0–174.5	42.4	3476–2949, 3110, 2943, 1740, 1628, 1238, 1141, 1000, 764	2.05–2.20 (m, 2H); 2.20–2.40 (m, 2H); 2.74–2.76 (m, 1H); 3.79 (m, 4H); 4.00 (s, 3H) 4.51 (s, 2H); 5.32 (s, 2H); 6.12 (s, 2H); 7.99 (s, 1H); 8.27 (s, 1H); 8.63 (s, 1H); 13.60 (s, 1H)	20.32, 36.54, 37.43, 51.24, 62.65, 78.28, 88.04, 123.79, 124.93, 135.09, 138.40, 202.91

[a] Melting points are uncorrected. **II** and **IIIa** were recrystallized from methanol, **I** and **IIIb** from ethanol-water and **IV** from methanol-ether
[b] Infrared spectra (KBr pellets) were obtained on a Perkin-Elmer FT-IR 1725 X spectrometer
[c] ^{1}H and ^{13}C NMR spectra were recorded on a Varian XL-GEM 300 spectrometer (in DMSO-d_6, internal Me_4Si)
[d] ^{1}H and ^{13}C NMR spectra were closely related to that of **IIIa**

Table 2 Second-order rate constants ($k \pm$ SE) for reaction of the substrate acetylthiocholine with the indicated compounds

Substrate (mM)	Compound (mM)	$k \pm$ SE (l/mol per min)
0.25–1.0	**II** 0.08–0.25	37 ± 4
0.05–1.0	**III** 0.20–1.0	27 ± 4
0.05–1.0	**IV** 0.10–1.0	23 ± 2

Constants were calculated according to Eq. 1 (see text). Each constant was obtained from measurements done with 6-11 substrate/compound concentration pairs

Table 3 Reversible inhibition of acetylcholinesterase by the indicated compounds measured with acetylthiocholine as substrate

Compound (mM)	Substrate (mM)	K_a (mM)	K_m (mM)
I 1–10	0.10–1.0	1.6 ± 0.2	0.45 ± 0.05
II 0.1–0.5	0.05–0.50	0.073 ± 0.011	0.34 ± 0.08
III 0.5, 1.0	0.05–0.50	0.24 ± 0.05	0.13 ± 0.03
IV 0.5–2.0	0.05–0.25	0.25 ± 0.04	0.073 ± 0.012
		K_i (mM)	K_{ss} (mM)
I 5, 10	1, 5, 10	4.8 ± 0.1	22 ± 7

Constants ± SE were calculated as described in the text. Each constant was derived from measurements done with 6–8 substrate/compound concentration pairs

$$c/t = k \cdot s \cdot i \quad (1)$$

where c is the released thiocholine concentration, t is the time of reaction, and s and i are the initial substrate and oxime concentrations. Initial concentrations were used, because the time of reaction was short and the decrease in concentration did not need to be taken into account.

It was shown by means of one-way analysis of variance and multiple-range Scheffé test (at the 95% confidence level) that the imidazolium oxime (**II**) reacted significantly faster with ATCh than its conjugates with 3-oxoquinuclidinium (**III** and **IV**); the latter two reacted about equally fast (Table 2). All three rate constants were of the same order as those of 2-PAM, Toxogonin and six other imidazolium or pyridinium oximes (Škrinjarić-Špoljar et al. 1992).

Reversible binding of compounds to acetylcholinesterase

All four compounds were reversible inhibitors of AChE. The enzyme/compound dissociation constants for binding to the catalytic site (K_a) were evaluated from the kinetics of enzyme inhibition at low substrate concentrations and calculated according to Aldridge and Reiner (1969, 1972) and Radić et al. (1984, 1991):

$$K_{app} = (v_i \cdot i)/(v_o - v_i) = K_a[1 + (s/K_m)] \quad (2)$$

K_{app} is the apparent enzyme/compound dissociation constant at a given substrate concentration (s), v_o and v_i are the enzyme activities measured at a given substrate concentration in the absence and in the presence respectively of a given inhibitor concentration (i). K_m is the Michaelis constant for the substrate. The K_a values obtained are given in Table 3. Compound **II** has the highest affinity (lowest K_a) and compound **I** the lowest affinity. The two 3-oxoquinuclidinium-imidazolium oximes (**III** and **IV**) have K_a values between those of compounds **I** and **II**.

3-Oxoquinuclidinium (**I**) does not react with acetylthiocholine, and inhibition by this compound could therefore be studied over a wide concentration range. At high substrate concentrations, the kinetics of AChE inhibition with compound **I** deviated from Eq. 2. This was attributed to allosteric binding of the quinuclidinium moiety. The corresponding dissociation constant for the allosteric site (K_i) was calculated from the linear relationship between K_{app} and substrate concentrations between 1 and 10 mM (Table 3). For allosteric binding, the intercept of the line (K_{app} vs s) with the ordinate corresponds to K_i and the intercept on the abscissa to K_{ss}, the substrate inhibition constant (Aldridge and Reiner 1969; Radić et al. 1991). Possible binding of **II**, **III** and **IV** to the allosteric site in AChE could not be studied, because at high substrate concentrations all three oximes induced the hydrolysis of ATCh at a rate which was equal to, or even higher than the rates of the enzymic hydrolysis, and thus interfered with the studied reaction.

The K_{ss} constant derived from the kinetics of inhibition with compound **I** (Table 3) was within the order known for the AChE/ATCh reaction (cf. Aldridge and Reiner 1972; Radić et al. 1991; Simeon 1974) thus supporting the conclusion that compound **I** binds to the allosteric site. The K_m values, derived from the kinetics of inhibition with all four compounds at low substrate concentrations, ranged from 0.073 to 0.45 mM (Table 3); these values are close to the published K_m for ATCh covering a range from 0.05 to 0.14 mM (cf. Aldridge and Reiner 1972; Radić et al. 1991; Simeon 1974). This supports the conclusion that binding constants derived from inhibition studies at low substrate concentrations correspond to binding of the inhibitors to the catalytic site of AChE.

Protection of acetylcholinesterase against phosphorylation by Soman and VX

All four compounds protected the enzyme against phosphorylation. Protection was expressed in terms of the protective index (PI) which corresponds to the ratio:

$$PI = k_a/k'_a \quad (3)$$

where k_a and k'_a are the first-order rate constants of phosphorylation in the absence and in the presence of the reversible inhibitor respectively. The measured PI values are given in Table 4. For each OP compound, PI

Table 4 Protection of AChE against phosphorylation by Soman (5 nM) and VX (20 nM)

Compound (mM)	Organo-phosphate	$PI_{measured}$	$PI_{calculated}$		
			Eq. 4	Eq. 5	Eq. 6
I 1.4	Soman	1.9	1.9	2.2	2.4
	VX	2.0			
I 6.0	Soman	9.0	4.8	6.0	10.7
	VX	7.0			
II 0.08	Soman	2.1	2.1		
	VX	2.0			
II 0.16	Soman	3.0	3.2		
	VX	3.0			
III 0.2	Soman	2.0	1.8		
	VX	1.9			
III 0.4	Soman	3.2	2.7		
	VX	4.1			
IV 0.1	Soman	2.0	1.4		
	VX	2.1			
IV 0.3	Soman	3.8	2.2		
	VX	4.2			

The protective indices are mean values of two experiments, except for **IV** where single experiments were done. The protective indices $PI_{calculated}$ were calculated according to the indicated equations using K_a and K_i values from Table 3; $PI_{calculated}$ value applies to any acylating inhibitor, because it is a ratio of acylating rate constants

was measured at two concentrations of the reversible inhibitor.

When a reversible inhibitor binds only to the catalytic site, the PI value is (Reiner 1986):

$$PI = 1 + i/K_a \quad (4)$$

When a reversible inhibitor binds to the catalytic and allosteric site, the PI value is either:

$$PI = 1 + i/K_a + i/K_i \quad (5)$$

or:

$$PI = (1 + i/K_a) \cdot (1 + i/K_i) \quad (6)$$

Eq. 5 holds for compounds which form two binary complexes with the enzyme (one at the catalytic site and the other at the allosteric site), while Eq. 6 holds for compounds which in addition form a ternary complex where both sites on the enzyme are simultaneously occupied by the reversible inhibitor (Reiner 1986).

The measured PI values for compound **II** agreed with PI values calculated from Eq. 4. For compounds **I**, **III** and **IV**, the measured PI values were the same as, or higher than those calculated from Eq. 4. The difference between measured PI and those calculated from Eq. 4 was more pronounced at higher concentrations of the reversible ligand. The higher PI values suggest that allosteric binding of the 3-oxoquinuclidinium derivative contributed towards the enzyme protection. Equations 5 and 6 could be verified only for compound **I** (Table 4). The measured **PI** values were higher than predicted from Eq. 5, but lower than predicted from Eq. 6. From this result one may only conclude that allosteric binding contributed to the enzyme protection, but the result was

Table 5 Acute toxicity (i.p. LD_{50}) of the tested compounds in male mice

Compound	LD_{50} (95% confidence limits)	
	(mg/kg)	(mmol/kg)
I	56[a]	0.21[a]
II	182 (154–217)	0.68 (0.58–0.81)
III	225[a]	0.49[a]
IV	283[a]	0.77[a]

[a] 95% confidence limits could not be calculated from the tables in Thompson (1947) and Weil (1952)

Table 6 Effect of the tested compounds combined with atropine upon (s.c.) Soman toxicity in male mice (*TF* Therapeutic factor, *TD* therapeutic dose)

Antidotes	Soman LD_{50} (μg/kg) (95% confidence limits)	TF[b]	TD[c]
None	220 (183–264)	–	–
Atropine only	247 (204–298)	1.12 (0.93–1.36)	0.79
I + atropine	495[a]	2.25[a]	2.00
II + atropine	438 (372–516)	1.99 (1.69–2.35)	1.58
III + atropine	525 (468–588)	2.38 (2.13–2.67)	2.00
IV + atropine	622[a]	2.83[a]	2.52

[a] 95% confidence limits could not be calculated from the tables in Thompson (1947) and Weil (1952)
[b] Calculated as: LD_{50} (Soman + antidote)/LD_{50} (Soman)
[c] Highest multiple LD_{50} of Soman fully counteracted by an antidote (survival of all animals)

ambiguous concerning which of the two equations is more applicable.

Effect of compounds on mice poisoned by Soman

All four compounds were toxic to mice (Table 5). Symptoms of poisoning (tremor, ataxia, dyspnoea, impaired movement) showed up 3–5 min after the i.p. administration. Animals died after 5–7 min or recovered after *c*. 20–30 min. Expressed on a molar basis, compound **I** was the most toxic and compound **IV** the least toxic. The four compounds, plus atropine, were tested as antidotes against Soman (Table 6). The compounds were administered at equitoxic doses and were given (i.p.) immediately after Soman (given s.c.). Animals developed mild central, nicotinic and muscarinic symptoms within 30–60 min. After that time all symptoms (salivation, fasciculation, ataxia, dyspnoea) became more pronounced and persisted for up to 3 h; tremor could be provoked for up to 24 h.

The therapeutic factors ranged from 1.99 to 2.83 LD_{50} of Soman. The LD_{50} of Soman (220 μg/kg) given alone was slightly lower than the LD_{50} of Soman with atropine (247 μg/kg; Table 6). TF values calculated on the basis of LD_{50} of Soman and atropine (not given in Table 6), were therefore negligibly lower than those based on the LD_{50} of Soman without atropine. The therapeutic dose (TD) of the tested compounds was between 1.58 and 2.52 LD_{50} of Soman while the therapeutic dose of atropine alone was 0.79 LD_{50} of Soman (174 μg/kg). All animals given Soman with no antidote survived at 0.72 LD_{50} of Soman (159 μg/kg). All four compounds had very similar antidotal effects (Table 6). However, compound **II** tended to be the least effective and compound **IV** was slightly more efficient than the other studied compounds.

Compounds **III** and **IV** differ only in the link between the 3-oxoquinuclidinium derivatives and the imidazolium oxime (Table 1). The $-CH_2-O-CH_2-$ link (**IV**) resulted in better antidotal properties than the $-(CH_2)_3-$ link (**III**). The oxime group seems not to be required for an antidotal effect. Compound **I**, with no oxime group, had the same TD as **III** (Table 6) and also the same TD as the 3-hydroxyimino-1-methylquinuclidinium, which was tested under the same experimental conditions (Lucić et al. 1997).

Some oximes seem to have pharmacological effects, which are not related to the reactivation of phosphorylated AChE (cf. Van Helden et al. 1996). Several quaternary and tertiary 3-substituted quinuclidinium derivatives were shown to inhibit choline uptake into the neuron, hence influencing the acetylcholine synthesis (Sterling et al. 1991, 1993). The antidotal effect of 3-substituted quinuclidinium derivatives against OP poisoning has not yet been elucidated.

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