Novel Nucleophilic Compounds with Oxime Group as Reactivators of Paraoxon-Inhibited Cholinesterases

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Received October 25, 2009: Revised December 14, 2009: Accepted December 14, 2009

Abstract: New cholinesterase reactivators are synthesized as potential antidotes for treatment of organophosphorus agent poisonings or as part of pseudo catalytic scavengers for improvement of the nerve agent prophylaxis. In this study, three novel potential cholinesterase reactivators (K064 - (*E*)-1-(pyridinium)-4-(2-hydroxyiminomethylpyridinium)-but-2-ene dibromide; K065 - (E)-1-(quinolinium)-4-(2-hydroxyiminomethylpyridinium)-but-2-ene dibromide; K066 - (E)-1-(isoquinolinium)-4-(2-hydroxyiminomethylpyridinium)-but-2-ene dibromide) were synthesized and tested for their potency to reactivate acetylcholinesterase (AChE, EC 3.1.1.3) and butyrylcholinesterase (BChE, 3.1.1.8) inhibited by pesticide paraoxon. As resulted, none from the synthesized compounds surpassed currently clinically used reactivators (pralidoxime, obidoxime and HI-6).

Keywords: Acetylcholinesterase, Butyrylcholinesterase, Nerve agent, Pesticide, Reactivator, Oxime, Paraoxon, Scavenger.

INTRODUCTION

Chemical warfare agents (CWA) pose extraordinary high threat if considered their misuse by terrorists. As known from the past, they were already misused by the Aum Shinrikjo sect in 1995 in Tokyo subway (so called Sarin Attack) [1]. Owing to the simple preparation and availability of precursors of these agents, they could be much more easily misused if compared with biological (delay in symptoms onset) or nuclear (relatively expensive) weapons. Nerve agents seem to be the most toxic group of CWA with the highest probability of use. Sarin, soman or VX agent are the most known members of the nerve agent family [2].

These agents cause an inhibition of cholinesterases which are then unable to hydrolyze endogenic esters in the human body - Fig. (**1**). Especially, inhibited acetylcholinesterase (AChE; EC 3.1.1.7) is unable to serve its physiological role – hydrolyzing the neuromediator acetylcholine at the synaptic clefts. This failure of nerve system leads to cholinergic crisis and death of the intoxicated organism [3].

There are several approaches to fight against the nerve agent intoxications. Several drugs are administered prior (prophylaxis) and several after (treatment) the nerve agent intoxication [4]. Among the prophylactic approaches, bioscavengers are considered to be most promising direction [5-7]. If the post-exposure treatment is discussed, AChE reactivators are the most important group of antidotes and therefore finding of the most universal AChE reactivator is of high interest [8-10].

In this communication, synthesis of three novel cholinesterase reactivators (K064, K065, K066) is described - Fig. (**2**). Their design was derived from the knowledge, that qiunolinium and isoquinolinium salts exert high AChE inhibition.[21] Furthermore, their potency to reactivate human AChE and butyrylcholinesterase (BChE; EC 3.1.1.8) inhibited by the pesticide paraoxon (*O*,*O*-diethyl *O*-4-nitro-phenyl phosphate) is described. Clinically used AChE reactivators, pralidoxime, obidoxime and HI-6, were used as appropriate standards for the comparison - Fig. (**3**).

MATERIALS AND METHODS

Chemicals

As shown in the Fig. (**2**), all newly prepared compounds (K064 - (*E*)-1-(pyridinium)-4-(2-hydroxyiminomethylpyridinium)-but-2-ene dibromide; K065 - (E)-1-(quinolinium)-4-(2-hydroxyiminomethylpyridinium)-but-2-ene dibromide; K066 - (E)-1-(isoquinolinium)-4-(2-hydroxyiminomethylpyridinium)-but-2-ene dibromide) are non-symmetric bisquaternary compounds with the oxime group which is responsible for the reactivation process - Fig. (**1**). Owing to this fact, stepwise synthetic approach *via* the monoquaternary intermediate is needed. The whole synthetic process is outlined in Fig. (**4**). To get the required monoquaternary intermediate, the first reaction was conducted at specific reaction conditions (excess of alkylating agent; very diluted solu-

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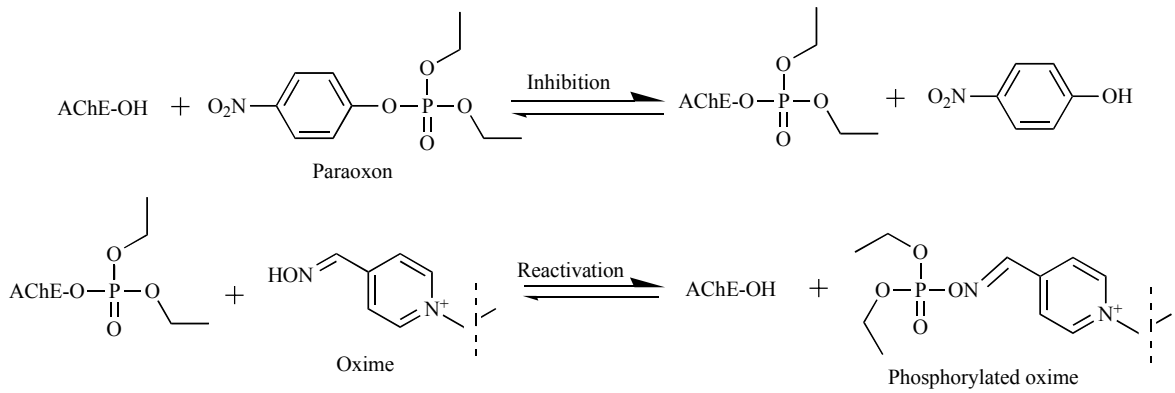

Fig. (1). AChE is inhibited immediately when paraoxon reacts with serine hydroxyl group in the enzyme active site. Reactivation occurs when oxime reactivator cleaves the bond between AChE and diethoxyphosphoryl moiety.

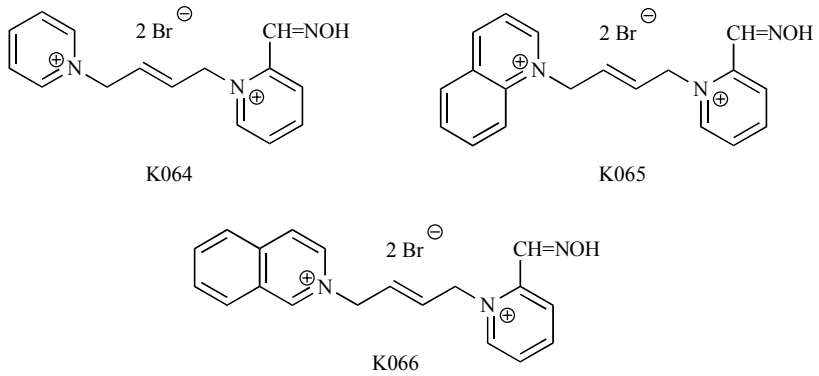

Fig. (2). Structures of newly prepared cholinesterase reactivators.

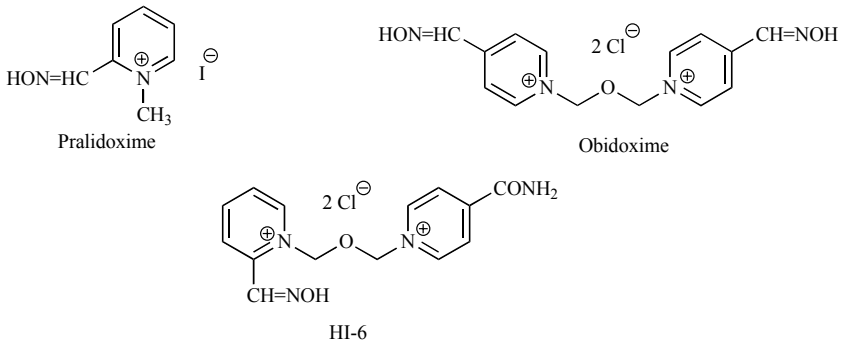

Fig. (3). Structures of clinically used AChE reactivators, currently used in the therapy of organophosphate agent poisonings.

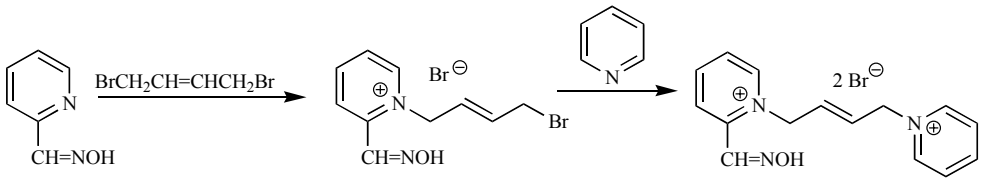

Fig. (4). General preparation of bisquaternary non-symmetric AChE reactivators.

tion). The exact description of this approach was described earlier by our group in Musilek *et al.* [11-13].

Yields of all reaction, melting points and NMR spectra of obtained products are summarized in Table 1. Both, 1H- and 13C-NMR spectra were recorded at 300 and 75 MHz, respectively, on a Varian Mercury 300 spectrometer, using D2O as solvent. As shown, the yields are in the range between 24-55 %, which is comparable with the yields obtained for similar compounds published earlier [11-13].

Purity of all tested AChE reactivators was tested using TLC (Merck; DC-Alufolien Cellulose F; mobile phase *n*-butanol : acetic acid : water – 5 : 1 : 2; detection by Dragen-

Table 1. Melting Points, Yields of all Reactions and NMR Spectra of Newly Prepared Compounds

Comp.	m.p.	Yield	NMR Spectra
K064	>300°C	55%	^{1}H NMR (δ, 300 MHz, D_2O) 5.43 (m, 2H, CH_2), 5.83 (m, 2H, CH_2), 6.06 (dm, 1H, *J* = 16 Hz, CH=), 6.37 (dm, 1H, *J* = 16 Hz, CH=), 8.04-9.35 (m, 9H, arom), 8.68 (s, 1H, CH=N). The signal of =NOH disappeared in deuterated solvent. ^{13}C NMR (δ, 75 MHz, D_2O) 47.59 (CH_2), 63.53 (CH_2), 126.73 (CH=), 126.76 (CH=), 128.72 (CH-3',5'), 129.03 (CH-3), 129.61 (CH-5), 142.18 (CH-2',6'), 146.03 (CH-6), 147.88 (CH-4), 148.13 (CH-4'), 149.25 (CH=N), 157.02 (C-2).
K065	>300°C	24%	^{1}H NMR (δ, 300 MHz, D_2O) 5.45 (m, 2H, CH_2), 5.93 (d, 2H, *J* = 8 Hz, CH_2), 6.09 (dm, 1H, *J* = 16 Hz, CH=), 6.29 (dm, 1H, *J* = 16 Hz, CH=), 8.02-9.35 (m, 11H, arom), 8.79 (s, 1H, CH=N). The signal of =NOH disappeared in deuterated solvent. ^{13}C NMR (δ, 75 MHz, D_2O) 48.23 (CH_2), 60.03 (CH_2), 118.40 (arom), 123.31 (arom), 123.58 (arom), 123.88 (arom), 127.12 (CH=), 127.33 (CH=), 128.55 (arom), 129.41 (arom), 129.54 (CH-3), 134.22 (CH-5), 139.64 (arom), 142.65 (arom), 144.46 (arom), 146.85 (CH-6), 148.38 (CH-4), 149.81 (CH=N), 157.26 (C-2).
K066	185-188°C	25%	^{1}H NMR (δ, 300 MHz, D_2O) 5.42 (d, 2H, *J* = 6.5 Hz, CH_2), 5.54 (d, 2H, *J* = 5.5 Hz, CH_2), 6.08 (dt, 1H, *J* = 16, 6.5 Hz, CH=), 6.37 (dt, 1H, *J* = 16, 5.5 Hz, CH=), 8.02-8.28 (m, 6H, arom H-5', H-4,5,6,7,8), 8.42 (d, 1H, *J* = 6 Hz, arom H-3'), 8.45 (d, 1H, *J* = 6 Hz, arom H-3), 8.54-8.60 (m, 1H, arom H-4'), 8.62 (s, 1H, CH=N), 8.87 (d, 1H, *J* = 6 Hz, arom H-6'), 9.77 (s, 1H, arom H-1). The signal of =NOH disappeared in deuterated solvent. ^{13}C NMR (δ, 75 MHz, D_2O) 48.42 (CH_2), 62.37 (CH_2), 127.53 (CH=), 127.62 (CH=), 128.64 (arom), 129.25 (arom), 129.62 (CH-3), 129.89 (arom), 132.33 (arom), 133.57 (CH-5), 139.59 (arom), 142.93 (arom), 146.54 (CH-6), 148.73 (CH-4), 149.18 (arom), 151.83 (CH=N), 157.22 (C-2).

dorff Reagent) and NMR (Varian Gemini 300, Palo Alto CA, USA) prior to their use [14-15]. Purity of all newly prepared compounds was 98 % and higher.

Paraoxon (POX; *O*,*O*-diethyl-*O*-4-nitrophenylphosphate) was purchased from Dr. Ehrenstorfer (Augsburg, Germany) in 95% purity. All other chemicals used in this experiment were of analytical grade and were purchased from Sigma Aldrich (Czech Republic).

In Vitro Reactivation of AChE and BChE

All the prepared compounds were tested for their activity to reactivate AChE and BChE inhibited by paraoxon. The whole method is described in Musilova *et al.* [16]. The blood samples were collected from healthy volunteers from the vein into a disposable syringe containing 3.8% sodium citrate (the ration blood/citrate was 1:10 w/w). The citrated blood was centrifuged for 20 min at 5000 rpm and the plasma was removed as supernatant, frozen and was kept under -80 ºC (source of BuChE). The erythrocytes were washed three times with phosphate buffer (PB; 0.1 M, pH 7.4) and then hemolyzed in PB (0.01 M, pH 7.4) in a ratio 1:10 (w/w), frozen and kept under -80 ºC (source of AChE) [16]. Human erythrocyte AChE or plasma BChE was inhibited by solution of paraoxon to 5 % of its original activity. Concentration of paraoxon, causing 95% enzyme inhibition (3.38×10^{-6} for AChE and 1.4×10^{-7} for BuChE) was calculated from experimentally founded IC_{50} values. The inhibition of AChE was started in plastic cuvette by addition of paraoxon solution in isopropanol to the mixture of phosphate buffer (0.05 M, pH 7.4) and hemolyzate (activity before inhibition was set to 10 U/L) or plasma (activity was set to 13.3 U/L) [16]. Then, inhibited enzyme was incubated for 10 min with solution of reactivator in phosphate buffer (0.05 M, pH 7.4) at concentration 10^{-4} and 10^{-5} M. Activity of AChE (BChE) was measured spectrophotometrically by modified method according to Ellman with acetylthiocholine (butyrylthiocholine) as substrate.[17] The final concentration of DTNB and acetylthiocholine or butyrylthiocholine in the mixture was 1 mM.[16] All results were corrected for hydrolysis of substrate by reactivators (oximolysis). Oximolysis was measured separately for each compound and concentration using the same reaction as for the determination of enzyme activity after reactivation, but hemolysate or plasma solution was replaced with the same volume of phosphate buffer (0.05 M, pH 7.4). Reactivation potency was calculated from the formula:

$$\%R = (1-(a_0-a_r)/(a_0-a_i))\times 100$$

where %R is percent of reactivation, a_0 is activity of intact enzyme, a_i is activity of inhibited enzyme and a_r is activity of reactivated enzyme minus oximolysis.

Each measurement was repeated three times and was conducted under standard laboratory temperature (25 ºC). Calculations were performed using software GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA (www.graphpad.com).

All experiments were carried out in compliance with the current law of Czech Republic.

RESULTS AND DISCUSSION

Reactivation results are shown in Fig. (**5**) and Table 2.

According to our results, the most potent reactivator of paraoxon-inhibited AChE from newly developed compounds (at both concentrations tested) was oxime K066. However, its reactivation ability did not surpassed 5 %. In case of paraoxon-inhibited BChE, the most promising compound was again reactivator K066. Reactivation ability of other tested oximes was below 1.5 %.

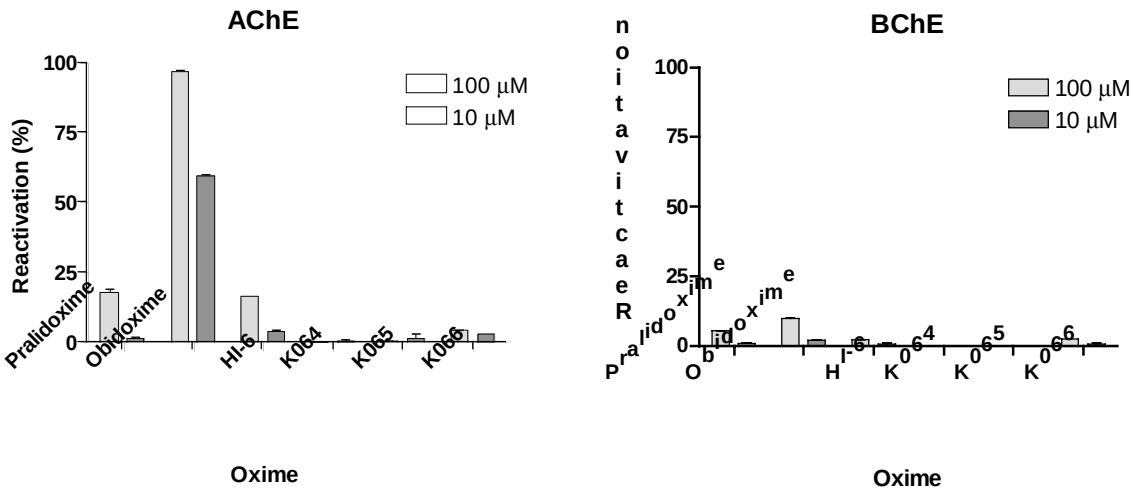

Fig. (5). Reactivation of paraoxon-inhibited AChE and BChE by newly prepared bisquaternary oxime reactivators.

Table 2. Potency of Tested Oximes to Reactivate Paraoxon- Inhibited Human Erythrocyte AChE and Plasma BChE at Concentrations 100 μM and 10 μM

	Reactivation (%)							
	AChE				BChE			
Concentration	100 μM		10 μM		100 μM		10 μM	
Reactivator	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Pralidoxime [18]	18.0	0.7	1.3	0.7	5.5	0.1	1.0	0.2
Obidoxime [18]	96.9	0.7	59.4	0.7	9.9	0.3	2.2	0.3
HI-6 [18]	16.1	0	3.9	0.7	2.3	0.2	0.8	0.4
K064	0	0	0.4	0.4	0	0	0	0
K065	0.2	0.2	1.5	1.5	0	0	0	0
K066	4.2	0.3	3.0	0	2.7	0	0.9	0.3

(%, mean value of three independent determinations, time of inhibition by paraoxon 120 min; time of reactivation by AChE reactivators - 10 min; pH 7.4; temperature 25 ºC).

It is clearly shown that no oxime prepared in this study is able to reach reactivation potency of standard reactivators for both AChE and BChE. If considered AChE reactivation, our results confirm the general fact, that obidoxime is at present time the most active reactivator in case of paraoxon poisoning [18,19]. On the contrary, currently the most discussed oxime – oxime HI-6 – should not be used as treatment of paraoxon because of almost no benefit. Similar results were published already earlier [8,18]. In case of BChE reactivation, only obidoxime was able to slightly reactivate BChE inhibited by paraoxon (cca 10 %). Reactivation activity of other tested reactivators was insufficient. The problem of BChE reactivation by clinically used reactivators was already discussed earlier [16,20].

All three novel compounds were, unfortunately, not good reactivators of AChE and BChE inhibited by paraoxon. As it is known from the former results, quaternary quinolines and isoquinolines exert high inhibition activity towards intact AChE [21]. This could be the main reason for their low reactivation potency of paraoxon-inhibited AChE and BChE. Moreover, results obtained in this study were focus on paraoxon-inhibited cholinesterases. If other pesticides or nerve agents will be used, probably different (perhaps promising) results could be expected. Owing to this, further evaluation of these oximes is recommended. Although our new reactivators did not surpass those clinically active ones, the need of novel structural variants (although with non-favorable results) is necessary to get the compact view on the structure-activity relationship to design in future a universal compound with broad reactivation activity.

ACKNOWLEDGEMENT

This work was supported by the MoD of the Czech Republic (project OVUOFVZ200902 - Novel Prophylactic Antidotes of Nerve Agent Poisonings Based on Scavengers).

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