Potency of Several Structurally Different Acetylcholinesterase Reactivators to Reactivate House Fly and Bovine Acetylcholinesterases Inhibited by Paraoxon and DFP

No-Joong Park, Young-Sik Jung,* Kamil Musilek,† Daniel Jun,† and Kamil Kuca†,*

Bioorganic Science Division, Korea Research Institute of Chemical Technology, P.O. Box 107, Yuseong, Daejeon 305-606, Korea
**E-mail: ysjung@krict.re.kr*
†Department of Toxicology, Faculty of Military Health Sciences, Trebesska 1575, 500 01 Hradec Kralove, Czech Republic
**E-mail: kucakam@pmfhk.cz*
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Eight structurally different acetylcholinesterase reactivators derived from currently commercially available oximes were tested for their potency to reactivate acetylcholinesterase inhibited by pesticide paraoxon (P) and DFP (D). Housefly AChE (F) and bovine red blood cell AChE (B) were used as the source of the cholinesterases. Ellman's method was taken to examine cholinesterases activity. The results show that four AChE reactivators are potent AChE reactivators, able to reach reactivation potency of more than 30% in all cases - PF, PB, DF and DB. Their reactivation potency was comparable with that of pralidoxime and even higher compared with that of HI-6, standard AChE reactivators currently available on the market.

Key Words : Cholinesterase, Reactivator, Oxime, DFP, Paraoxon

Introduction

Organophosphorus (OP) compounds are used in agriculture as pesticides (parathion, chlorpyrifos), as drugs (metriphonate) and as military weapons, most notably as the nerve agents sarin, and soman.[1] The biological effects of these substances are mostly connected with the inhibition of the enzyme acetylcholinesterase (AChE; EC 3.1.1.7), which is a very important enzyme in the human body, splitting the neuromediator acetylcholine (ACh) at the synaptic clefts.[2] Due to this inhibition, AChE is unable to fulfill its physiological function. ACh accumulates at the synaptic clefts, over stimulates nerve receptors, and cholinergic crisis occurs. Intoxicated organism could die from respiratory insufficiency within minutes after exposure.[3]

Generally, anticholinergics such as atropine and AChE reactivators are used as first aid antidotes in the case of such intoxications.[1] AChE reactivators spur the antidotal effect of anticholinergics. These compounds break down the bond between inhibitor and enzyme and due to this liberate AChE, which is then able to fulfill its physiological role.[4]

There is a need for a specific AChE reactivator in the case of specific AChE inhibitor in contrast to anticholinergics, which can be administered automatically. Although there are a huge number of potential AChE reactivators, no single one of them can reactivate all OP inhibition regardless of inhibitor structure. Because of this fact, many laboratories throughout the world are searching for a new broad spectrum AChE reactivator.[5] Currently, the most promising AChE reactivator, HI-6, is designed a partially broad spectrum reactivator because of its low efficacy to reactivate tabun and pesticides-inhibited AChE.[6] Also pralidoxime,[7] the gold standard of AChE reactivators presently used as an antidote in the U.S., is a very poor reactivator for nerve-agent-inhibited AChE.[6b,8] Some AChE reactivators including HI-6 are *in vivo* effective against soman and tabun poisoning.[9] Moreover, similar protection against nerve agents was reported with non-oxime containing pyridinium salts (compound SAD-128).[10] These results indicate that bisquaternary compounds have an additional therapeutic action *in vivo*, which is not related to the AChE reactivation. One possible mechanism of this effect is that the oximes may block the ion channel associated with the nicotinic acetylcholine receptor and thus counteract the effects of excessive cholinergic stimulation.[11]

In the present work, our interest is in the evaluation of eight structurally different oximes (Figure 1) as reactivators for paraoxon and diisopropyl fluorophosphates (DFP) inhibited AChE. These reactivators were formerly synthesized at the Department of Toxicology Faculty of Military Health Sciences, The Czech Republic, and according to our previous results, seemed to be promising reactivators of both nerve agents- and pesticides-inhibited AChE.[12] For standard AChE reactivators, we refer to pralidoxime and HI-6, which are currently commercially available.

Material and Methods

Chemicals. All pyridinium oximes tested were prepared earlier at the Department of Toxicology Faculty of Military Health Sciences, University of Defense, The Czech Republic, using standard synthetic pathways.[5b,c,12a-c] Oxime HI-6 was prepared earlier at the Medicinal Science Division, Korea Research Institute of Chemical Technology, Korea, and 2-PAM was purchased from Sigma-Aldrich. DFP and paraoxon were commercially available from Fluka and Sigma-Aldrich,

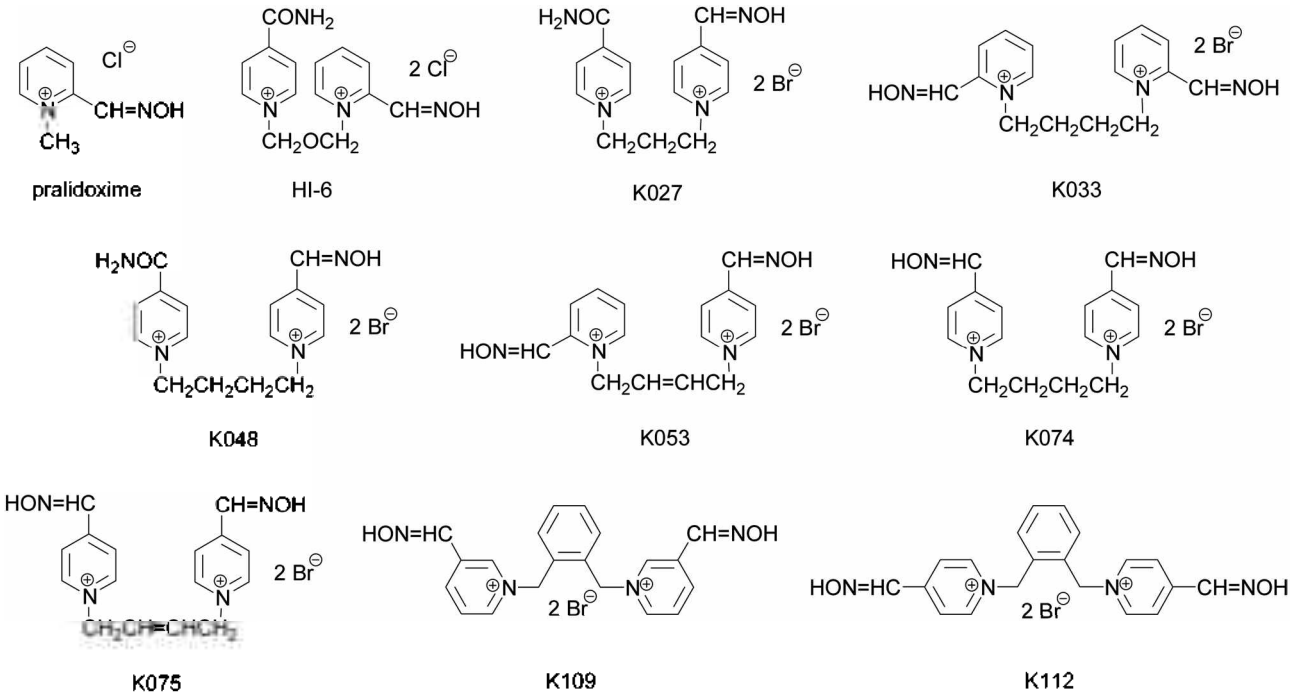

Figure 1. Structures of tested AChE reactivators.

respectively. The two kinds of AChE used in this experiment were obtained as follows. The first was extracts from housefly head from the Central Research Center, National Agricultural Cooperative Federation, Korea, and the second was bovine red blood cells (RBC) AChE, which was purchased from Sigma-Aldrich.

***In vitro* method**

Preparation of inhibited AChE: Housefly AChE was dissolved in 10 mL of phosphate buffer (pH 7.0, 0.1 M) and bovine RBC AChE in Tris-HCl buffer (pH 7.8, 0.1 M containing 1% Triton X-100). The AChE solutions were then incubated with paraoxon or DFP and paraoxon for 10 min at room temperature (1 mL enzyme + 10 uL inhibitor) to reach 99% inhibition of AChE activity, respectively. Afterwards, the inhibited-AChE was mixed with hexane (2 volumes), and centrifuged at 3,000 g at 4 °C for 3 min to remove surplus inhibitor. The aqueous phase was then separated and placed in ice bath.

Reactivation of inhibited AChE: 225 μL of inhibited AChE was mixed with 25 μL of 50 mM of the tested reactivator at the appropriate concentration in distilled water and incubated at 37 °C for 1 hour. Small molecules, such as the reactivator and phosphorylated oxime, were removed by gel-filtration through a micro spin-column packed with Sephadex-G50 (Bio-Rad).[13] The enzyme activity was measured in a 96-well Microplate using a microplate reader (Benchmark Microplate Reader, BioRad) at 415 nm and 37 °C with acetylthiocholine (1 mM) as substrate and DTNB (1 mM) as chromogen in 0.05 M Tris-HCl buffer, pH 7.8 with a slight modification of Ellman's AChE assay method.[14] For RBC AChE, 1% of Triton X-100 (Sigma-Aldrich), we added a Tris-HCl buffer to preserve enzyme activity. The AChE activity of the filtrate was measured in a 96-well microplate, and the percentage reactivation of AChE activity was calculated with the change of optical density per minute (OD/min) after correction with the control reaction, comparing it to that of the non-inhibited AChE.

Table 1. Potency of AChE reactivators to reactivate DFP and paraoxon-inhibited AChE

Sample No.	% Reactivation of AChE							
	HF-AChE				RBC-AChE			
	DFP		Paraoxon		DFP		Paraoxon	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
K027	63.2	0.71	59.6	0.44	95.3	3.17	31.6	1.96
K033	33.3	0.34	16.8	0.19	52.5	3.11	29.7	1.62
K048	48.3	0.64	32.6	0.58	73.8	2.86	40.8	1.25
K053	53.2	0.52	42.8	0.69	69.3	3.37	31.7	1.90
K074	41.2	0.53	27.3	0.46	74.8	3.10	26.1	1.76
K075	47.5	0.69	33.1	0.61	72.3	2.81	36.9	1.47
K109	19.1	0.20	9.6	0.09	9.2	2.84	20.5	1.24
K112	20.2	0.23	19.8	0.25	39.8	2.76	36.2	1.70
2-PAM	83.6	1.01	62.3	0.49	42.6	2.36	47.3	0.70
HI-6	8.3	0.24	10.2	0.07	18.7	3.38	41.6	0.78

Results

All results obtained are summarized in Table 1. Their graphical visualization is shown in Figure 2 (DFP) and Figure 3 (paraoxon).

As can be clearly seen in the case of DFP, the worst reactivation potency was achieved for HI-6 for both kinds of

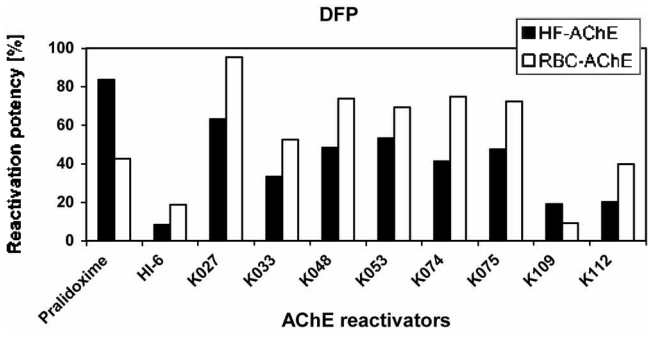

Figure 2. Potency of AChE reactivators to reactivate DFP-inhibited AChE (Source of enzyme – housefly and bovine RBC; inhibitor – DFP; time of inhibition – 10 min; time of reactivation – 60 min).

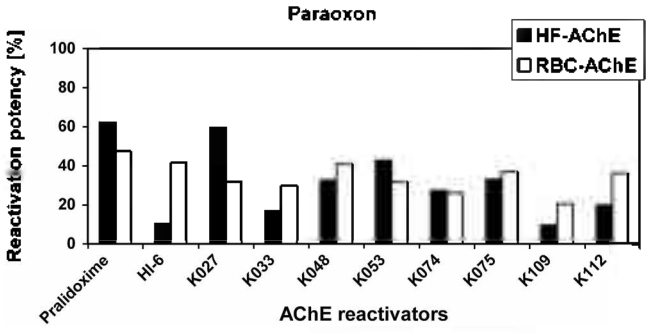

Figure 3. Potency of AChE reactivators to reactivate paraoxon-inhibited AChE (Source of enzyme – housefly and bovine RBC; inhibitor – paraoxon; time of inhibition – 10 min; time of reactivation – 60 min)

AChE. The second reactivator with similarly poor reactivation potency as HI-6 seems to be compound K109. The reactivation potency of both of these oximes does not surpassed 20%. On the contrary, other oximes tested achieved promising reactivation potency, reaching even 95% in the case of K027.

For paraoxon, the results were nearly similar. For housefly AChE, the lowest reactivation potency was achieved for K109, HI-6, K033 and K112. Contrary to these results, in the case of bovine AChE, the only oxime K109 reached poor reactivation potency.

Discussion

As has been shown many-times, the potency of currently available reactivators to reactivate pesticide-inhibited AChE is insufficient.[4,6a,b] This fact is confirmed not only experimentally, but also clinical praxis.[15] Due to the fact that all currently available AChE reactivators were formerly developed as antidotes against nerve agents, their efficacy in the case of pesticide poisoning is insufficient. For example, currently the most promising reactivator of nerve agents-inhibitions, oxime HI-6, is a poor reactivator of pesticide inhibitions.[5b,16] Only the time-tested reactivators, trimedoxime and obidoxime, seem to be still helpful for this purpose.[5b,c] Unfortunately, due to their toxicity described by some authors, their clinical use is limited.[17] Therefore, the development of new reactivators not only as nerve agent-antidotes but also for treatment of pesticide-intoxications is needed.

In the present work, eight structurally different AChE reactivators were tested for their potency to reactivate DFP and paraoxon-inhibited AChE from different sources (housefly and bovine). The results show all reactivators tested are able to reactivate inhibited AChE. But reactivation potencies differ depending on inhibitor and reactivator structure. This result is in good agreement with former results describing the same conditions.[18] Also, reactivation potency varied between the two enzyme sources, with the greatest difference observed being from the results of K027. The difference seems to be caused by the biochemical distinction between AChE sources with dissimilar kinetic properties, as described by Schwarz,[19] with distinct responses of oxime reactivation on various human AChE variants. In the case of DFP-inhibited RBC-AChE test, the reactivation activity of K027 was two times higher if compared with that of pralidoxime, which is involved in many countries as antidote for the first aid in the case of OP intoxications. In the case of paraoxon-inhibited RBC-AChE test, reverse phenomenon was obtained. These findings are due to the different structure of inhibitor used. To get better view on this problem, further investigations including molecular modeling study and *in vivo* study is needed.

As shown, currently the most promising reactivator, HI-6, is a poor reactivator of both pesticides. Our data confirm the previous results obtained.[5b,c] On the contrary, according to our results, pralidoxime presently regarded as an obsolete reactivator seems to when joined with oxime K027 the most potent reactivator of both organophosphorus compounds tested.

Because a relatively high number of AChE reactivators were tested, the structure-activity relationship of AChE reactivators can be discussed. As is generally known, there are several structural factors influencing the reactivation potency of AChE reactivators.[18] The main structural factors – presence and number of quaternary nitrogens, number and presence of oxime groups, length and shape of connecting chains between two pyridinium rings - should be mentioned. As the present study shows, although all tested compounds are potent as AChE reactivators, they can display marked differences in their reactivation potency.

Perhaps the most visible factor, which we discuss in this article, is the position of the oxime group at the pyridinium rings. As generally known, oximes in position three are the worst reactivators of nerve agents and pesticides.[18] Our results confirm this rule. According to other previous results, reactivators with an oxime group in position four are considered the most potent reactivators in the case of pesticide poisonings. The potency of reactivators with an oxime group in position two is in the middle, between the positions four and three. All reactivators tested in this work, except pralidoxime, confirm this fact.

Surprisingly, the reactivation effect we observed differs from former results obtained for other nerve agents and

pesticides. Future studies should address such deviations on the molecular level, using molecular modelling.

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