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Potency of new structurally different oximes to reactivate cyclosarin-inhibited human brain acetylcholinesterases

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Abstract

Antidotes currently used for organophosphorus pesticide and nerve agent intoxications consist of anticholinergics (atropine mainly) and acetylcholinesterase (AChE, EC 3.1.1.7) reactivators called oximes. Owing to the wide-spread of these toxic compounds worldwide, development of antidotes in the case of first aid is needed. To select the most promising AChE reactivators is a very time consuming process, which is necessary before approval of these compounds to be used as human antidotes. Because of ethical reasons, many developing experiments have been conducted on laboratory animals. However, these results often could not be transferred directly to human. Here, we have tested five newly developed AChE reactivators – K027, K033, K048, K074 and K075, which showed promising reactivation activity on rodents, as reactivators of inhibited human brain cholinesterases. For this purpose, cyclosarin was used as member of the nerve agent family. Oxime HI-6 and pralidoxime were used as AChE reactivator standards. Two AChE reactivators, K027 and K033, achieved comparable reactivation potency as HI-6. Moreover, oxime K033 reached its maximal reactivation potency at the lowest concentration which could be attained in humans.

Keywords: *Cholinesterase, reactivator, cyclosarin, human brain, nerve agent, inhibition*

Introduction

Cholinesterase reactivators are compounds generally used as prophylactics or therapeutic drugs for organophosphorus (OP) pesticide or nerve agent intoxications. The target of these drugs is acetylcholinesterase (AChE, EC 3.1.1.7) covalently bound to the OP inhibitor. These compounds are able break down the enzyme-inhibitor complex and subsequently liberate the free enzyme which is then able to serve its physiological role in the organism [1].

Due to the broad spectrum of OP pesticides and nerve agents, intoxications with these substances are well documented [2,3]. Owing to the fact that there exists no single AChE reactivator able to reactivate OP-inhibited AChE regardless of inhibitor structure [4], development of AChE reactivators still continues [5–8].

Before preclinical and clinical studies, the regular development process consisted of several *in vitro* and in vivo experiments conducted on laboratory animals mostly rodents such as mice, rats and guinea pigs [9,10].

There exists a correlation for *in vivo* toxicity of nerve agents and their *in vitro* anticholinesterase potency, however, the correlation is usually expressed within one species [11]. Some attempts have been made for the same correlation within different species [1]. For the treatment using reactivator, this correlation (reactivation potency *in vitro* vs therapeutic efficacy in vivo) is more complicated and can only be applied within one species. Consequently, the studies are focused on AChE from a human source (preferably brain) [10].

Due to interspecies differencies between laboratory animals and humans, in this work we wanted to test the reactivation potency of five newly developed AChE reactivators – oximes K027 (1-(4-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium) propane dibromide), K033 (1,4-bis(2-hydroxyiminomethylpyridinium)butane dibromide), K048

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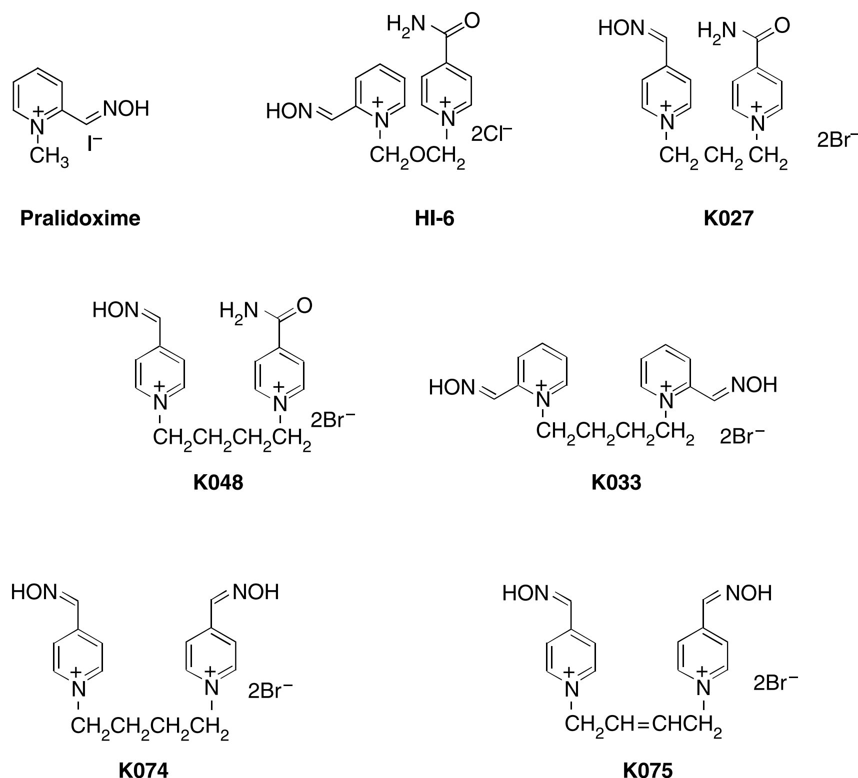

Figure 1. Structures of tested acetylcholinesterase reactivators.

(1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium)butane dibromide), K074 (1,4-bis(4-hydroxyiminomethylpyridinium)butane dibromide) and K075 (1,4-bis(4-hydroxyiminomethylpyridinium)but-2-ene dibromide) – to reactivate human brain cholinesterases inhibited by cyclosarin (Figure 1). These reactivators were selected according to our previous *in vitro* and *in vivo* results - all these newly developed AChE reactivators seemed to be promising reactivators of pesticides and nerve agent-inhibited AChE [12–18]. We needed to know if these reactivators were as promising as they were in the case of rat brain AChE reactivation. The gold standard for AChE reactivators, pralidoxime, and currently the most promising oxime, HI-6, were taken as compounds for comparison.

Material and methods

All newly developed oximes were prepared previously at our Department. Their structures and structure of pralidoxime and HI-6 are shown in Figure 1. The purity of all these reactivators was confirmed using TLC (DC-Alufolien Cellulose F; Merck, Germany; mobile phase BuOH-CH_3COOH-H_2O 5: 1: 2; detection by a solution of Dragendorff's reagent) and NMR (Varian Gemini 300; ^{1}H 300 MHz, ^{13}C 75 MHz, Palo Alto CA, USA) prior to their *in vitro* testing. Nerve agent (cyclosarin; GF; *O*-cyclohexylmethylflourophosphate) was obtained from the Military Facility Brno (97% purity). All other chemicals used were of reagent grade (Sigma-Aldrich, Czech Republic).

The whole study was done under the supervision of the Ethics Committee of the Faculty of Military Health Sciences in Hradec Kralove, Czech Republic. Human brain (nucleus caudatus part) homogenate (1/10 - w/v) was chosen as the source of the enzyme. Reactivation efficacy of the oximes used was tested *in vitro* on the model of AChE-inhibited by cyclosarin using the standard reactivation test with electrometric instrumentation [15]. The human brain homogenate (0.5 mL) was mixed with 0.5 mL of 0.01 μM cyclosarin in dry isopropanol and then incubated for 30 min at room temperature to give 95% inhibition. Then cyclosarin-inhibited AChE was incubated 10 min with a solution of reactivator (1 mL) of a predetermined concentration. Afterwards, 2.5 mL of 3M NaCl was added and distilled water added to a volume of 23 mL. Then, 2 mL of 0.02 M acetylcholine bromide was added and enzyme activity was measured titrimetrically at pH 8.0 and 25°C on the Autotitrator RTS 822 (Radiometer, Denmark). The whole method has been described previously [15].

Results

All obtained kinetic constants characterizing *in vitro* reactivation potency of tested AChE reactivators were calculated using our own computer program for non-linear regression [15]. They are summarized

Table I. Kinetic constants for cyclosarin-inhibited AChE reactivation

	K_R [μM]	k_R [min^{-1}]	k_r [min^{-1}. M^{-1}]
Pralidoxime	1259	0.04	32
HI-6	2	0.07	35000
K027	10	0.09	9000
K033	3	0.11	36667
K048	1000	0.03	30
K074	12589	0.03	2
K075	2512	0.02	8

K_R - dissociation constant of inhibited enzyme-reactivator complex.
k_R - the first-order rate constant of reactivation.
k_r - the second-order rate constant of reactivation (obtained as ratio k_R/K_R).

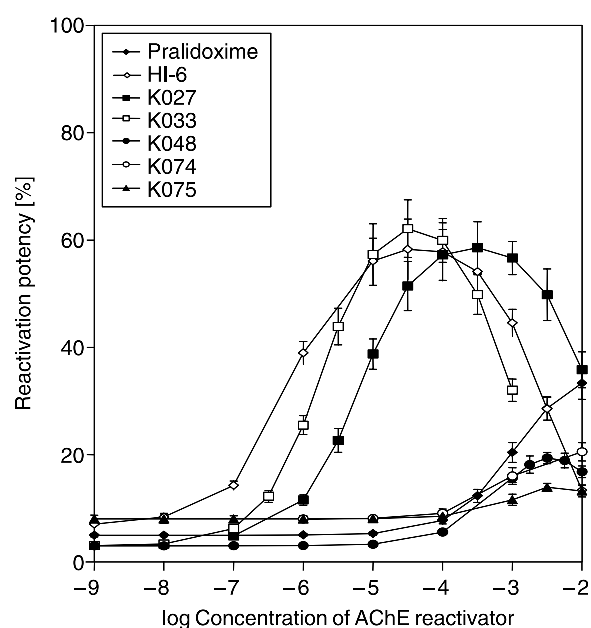

Figure 2. Concentration-reactivation potency relationship of tested acetylcholinesterase reactivators to reactivate cyclosarin-inhibited human brain cholinesterase *in vitro* (source of enzyme – human brain homogenate; time of inhibition – 30 min; time of reactivation – 10 min; pH 7.6; 25°C).

in Table I. The concentration-biological activity relationship for the reactivators is shown in Figure 2.

As it can be clearly seen, the highest reactivation affinity towards inhibited AChE was obtained for oximes HI-6 and K033. All other tested oximes had a lower affinity towards the cyclosarin-inhibited AChE. The first order kinetic constant k_R, characterizing the splitting of the bond between enzyme and inhibitor, was the highest for the newly developed oxime K033, followed by K027 and HI-6. The second order kinetic constant k_r, obtained as ratio k_R/K_R and characterizing the whole reactivation process, favours oximes HI-6 and K033.

As shown in Figure 2, all tested reactivators achieved a reactivation potency higher than 10%. The highest reactivation potency was reached for oxime K033, followed by K027 and HI-6. All the other oximes tested did not reach a promising reactivation potency. As can be clearly seen from the reactivation plot, the maximal reactivation potency of K033 and HI-6 was reached almost at the same concentration which is probably attainable for humans. From this point of view, oxime K027 did not reach as well the reactivation potency of the oximes HI-6 and K033.

The obtained reactivation curves are in several cases (pralidoxime, HI-6, K027 and K033) bell-shaped. This shape is due to two antagonistic processes – reactivation (the first increasing part of the reactivation curve) and reactivation-inhibition (the second decreasing part of the reactivation curve). The first part is caused by the reactivation process only. The second part is caused not only by the reactivation but also by the inhibition of AChE by the reactivator itself, since reactivators in high doses inhibit AChE.

Discussion

Development of potential reactivators for treatment of pesticide and nerve agents poisoning consists of several steps including reactivation efficacy *in vitro* and antidotal action *in vivo* [9]. For these studies, mostly laboratory animals are used [12,18]. However, results obtained for animals are not so easily transformed to humans.

Here, we wanted to know if our preliminary results obtained with laboratory animals were in good relationship with those obtained on human tissue. According to our results, oximes K033 and HI-6 seem to be the most promising reactivators of cyclosarin-inhibited AChE. Almost the same data were obtained on rat brain homogenate [13]. On the contrary, the third promising reactivator K027 was a poor reactivator of cyclosarin-inhibited AChE *in vitro* on rat brain tissue [16]. This fact supports previously obtained results, that species differences influence the reactivation process [10,19–21]. From this result we have to conclude, that species differences could disfavour some AChE reactivators in preliminary tests on laboratory animals. Due to this fact, screening *in vitro* should be conducted also on the human tissue enzyme.

Our results also confirmed the general understanding that AChE reactivators with the oxime group in the 2-position are the best reactivators of cyclosarin-inhibited AChE [4,22,23]. Oxime K027 is also in this case an exception, since its oxime group is placed in the 4-position of the pyridinium ring. The low reactivation potency of pralidoxime in comparison with HI-6 and K033 (reactivators with the oxime group in the 2-position) is probably due to the absence of the second pyridinium ring in its molecule. It is generally believed that bisquaternary oximes have

higher affinity towards both intact and inhibited AChE compared to monoquaternary ones [4].

In conclusion, screening for selection of more effective AChE reactivators could be misleading if it is performed on AChE obtained from animal tissue only. Therefore, *in vitro* tests using human brain AChE are to be considered as very important and valuable.

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