Journal of Enzyme Inhibition and Medicinal Chemistry, December 2003 Vol. 18 (6), pp. 529–535

Taylor & Francis
healthsciences

A Comparison of the Ability of a New Bispyridinium Oxime—1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium)butane Dibromide and Currently used Oximes to Reactivate Nerve Agent-inhibited Rat Brain Acetylcholinesterase by *In Vitro* Methods

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(Received 26 May 2003; In final form 13 June 2003)

The efficacy of a new bispyridinium oxime 1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium)-butane dibromide, called K048, and currently used oximes (pralidoxime, obidoxime, the oxime HI-6) to reactivate acetylcholinesterase inhibited by various nerve agents (sarin, tabun, cyclosarin, VX) was tested by *in vitro* methods. The new oxime K048 was found to be a more efficacious reactivator of nerve agent-inhibited acetylcholinesterase than pralidoxime (in the case of VX, tabun and cyclosarin), obidoxime (cyclosarin and tabun) and HI-6 (tabun) but it did not reach the efficacy of currently used oximes for the reactivation of acetylcholinesterase inhibited by sarin. Thus, the oxime K048 seems to be a relatively efficacious broad spectrum acetylcholinesterase reactivator and, therefore, it could be useful for the treatment of a nerve agent-exposed population if information about detection of the type of nerve agent is not available.

Keywords: Sarin; Cyclosarin; Tabun; VX; K048; Obidoxime; Pralidoxime; HI-6

INTRODUCTION

The highly toxic organophosphorus compounds, known as nerve agents (sarin, soman, tabun, cyclosarin or agent VX), belong to the most dangerous group of chemical warfare agents. Their acute toxicity is based on the irreversible inhibition of the enzyme—acetylcholinesterase (AChE; EC 3.1.1.7) and subsequent accumulation of the neuromediator acetylcholine at peripheral and central cholinergic sites.[1,2] AChE plays a key role in the physiological function of the cholinergic nervous system and, therefore, its inhibition is life-endangering. The inhibitory effect is based on phosphorylation or phosphonylation of the serine hydroxy group at the esteratic site of the active site of the enzyme.

The antidotal treatment of acute poisoning with nerve agents is based on the administration of anticholinergic drugs to antagonize the overstimulation of cholinergic receptors caused by accumulated acetylcholine and acetylcholinesterase reactivators to reactivate phosphonylated or phosphorylated AChE.[3] According to experimental data, quaternary pyridinium aldoximes have been found to be the most promising to reactivate nerve agent-inhibited AChE.[4] Monoquaternary pralidoxime (2-PAM, 2-hydroxyiminomethyl-1-methylpyridinium chloride) or more extended bisquaternary compounds such as obidoxime [1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxa-propane dichloride] and H-oxime HI-6 [1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)–2-oxa-propane dichloride] are the leading representatives of these aldoximes (Figure 1).

Unfortunately, currently used oximes are not sufficiently effective against some nerve agents and are not able to reactivate nerve agent-inhibited AChE regardless of the type of nerve agent used.[5–7] While pralidoxime and obidoxime have very low potency

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ISSN 1475-6366 print/ISSN 1475-6374 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/14756360310001605552

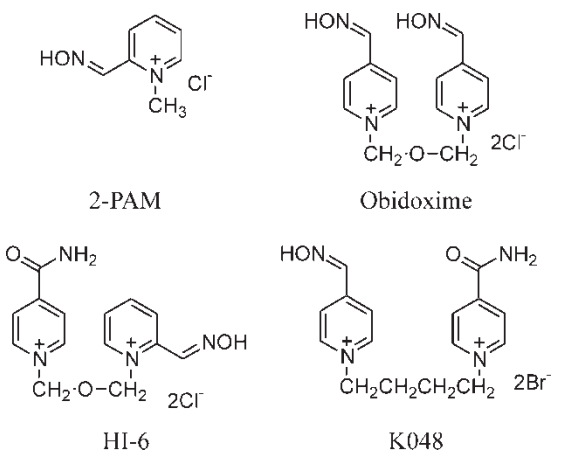

FIGURE 1 Chemical structure of AChE reactivators.

to reactivate soman and cyclosarin-inhibited AChE,[8,9] the oxime HI-6 seems to be practically ineffective to reactivate tabun-inhibited AChE.[10] Therefore, to find another bispyridinium oxime able to sufficiently reactivate nerve agent-inhibited AChE regardless of the type of nerve agent is still a very important task to improve the efficacy of antidotal treatment of acute poisonings with nerve agents. The present study compares the reactivating efficacy of currently used oximes (pralidoxime, obidoxime, HI-6) and new asymmetric bisquaternary oxime, called K048 1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium)butane dibromide, synthesized in our Department of Toxicology, against AChE inhibited by selected nerve agents (VX, tabun, sarin, cyclosarin) by *in vitro* methods.

MATERIALS AND METHODS

Animals

Male albino Wistar rats weighing 180–200 g were purchased from Biotest Konárovice (Czech Republic). They were kept in an air-conditioned room (22 ± 1°C and 50 ± 10% relative humidity, with lights from 07.00 to 19.00) and were allowed free access to standard food and tap water *ad libitum*. Experiments were performed under the supervision of the Ethics Committee of the Purkyně Military Medical Academy, Czech Republic.

Enzymes and Chemicals

After exsanguination of anaesthetized animals by decapitation (i.p. injection of urethane 1.5 g/kg), the rat brains were removed and used as a source of AChE after homogenization. All nerve agents (VX, tabun, sarin, cyclosarin) were obtained from the Military Technical Institute (Brno, Czech Republic) and were 89–95% pure. The purity was evaluated by acidimetric titration. The monopyridinium and bispyridinium oximes (pralidoxime, obidoxime, HI-6) including new oxime K048 were earlier synthesized at the Department of Toxicology of the Military Medical Academy (Czech Republic). Their purities were analysed using a HPLC technique. All other drugs and chemicals of analytical grade were obtained commercially and used without further purification.

In Vitro Experiments

Reactivation effectiveness of the oximes was tested *in vitro* on the model of AChE inhibited by VX agent, tabun, sarin or cyclosarin using a standard reactivation test with electrometric instrumentation.[11–12]

Determination of Percentage of Reactivation

The percentage of reactivation (%R) was calculated from the measured activities of the intact enzyme a_0, nerve agent-inhibited enzyme a_i, and reactivated enzyme a_r, using equation (1). The enzyme activity was measured at room temperature (25°C) and pH 7.6.

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

Determination of Activity of Intact AChE (a_0)

Rat brain homogenate (0.5 ml) was added to a 0.3 M sodium chloride solution (20 ml). Then 0.02 M solution of acetylcholine iodide (2.0 ml) was added and the system was adjusted with 0.3 M sodium chloride solution to a total volume of 25.0 ml. The liberated acetic acid was titrated with 0.01 M sodium hydroxide on an RTS 822 titrator in the pH-stat mode (pH 8.0) at room temperature (25°C). The ionic strength of the solution was adjusted with sodium chloride to at constant value I = 0.25 M. The slope of the linear part of the time-dependence of the sodium hydroxide used curve represents the activity of the intact enzyme (in fact, the initial rate of the enzymatic reaction).

Determination of Activity of Inhibited AChE (a_i)

Rat brain homogenate (0.5 ml) was treated with $5 \cdot 10^{-8}$ M aqueous solution of nerve agent (0.5 ml) for 30 min, which resulted in about 85% inhibition of the enzyme. 0.02 M solution of acetylcholine iodide (2.0 ml) was added and the mixture was adjusted to 25.0 ml with 0.3 M sodium chloride solution. The activity of the inhibited enzyme (a_i) was immediately determined in an analogous manner to that described in the previous experiment.

Determination of Activity of Reactivated AChE (a_r)

Reactivation of the enzyme, inhibited in the above mentioned experiment, was performed immediately after the inhibition. A solution (1.0 ml) of the reactivator was added to the enzyme in an appropriate concentration in the range $1{\cdot}10^{-7}$–$1{\cdot}10^{-2}$ M. After 10 min reactivation at 25°C, 0.02 M solution of acetylcholine iodide (2.0 ml) was added. The mixture was adjusted to 25.0 ml with 0.3 M sodium chloride solution and immediately afterwards the activity of the reactivated enzyme was determined in an analogous manner to that described in the previous experiments. According to our observation, the possibility of nerve agent-induced inhibition of released free enzyme during the reactivation of the enzyme is negligible.

Calculation of Rate and Dissociation Constants

The measured values of enzyme activity in relationship to the used concentrations of reactivator were used for the calculation of rate and dissociation constants using equation (2):

$$\%A = (100*(1 - EXP(-t*k_R*C_R/(C_R + K_R)))*(1 - a_i/a_0) + 100*a_i/a_0)/(1 + (C_S/K_M + 1)*C_R/(K_{dis}*f_d)) \quad (2)$$

where: %A = percentage of enzyme activity after reactivation, when 100% = activity of intact enzyme, t = time of the reactivation, k_R = pseudofirst order-rate constant of reactivation for $C_R = \infty$, C_R = molar concentration of reactivator, K_R = dissociation constant of the enzyme-inhibitor-reactivator complex, a_i = activity of inhibited enzyme, a_0 = activity of intact enzyme, C_S = molar concentration of substrate, K_M = dissociation constant of enzyme–substrate complex (Michaelis constant), K_{dis} = dissociation constant of enzyme–reactivator complex, f_d = dilution constant calculated from the ratio of volume of substrate reaction and volume of reactivator reaction.

The first part of the above equation $(1 - EXP(-t*k_R*C_R/(C_R + K_R)))$ describes the increase in enzyme activity due to dephosphorylation of the enzyme and the second part $(1 + (C_S/K_M + 1)*C_R/(K_{dis}*f_d))$ describes the decrease in enzyme activity due to a competitive reaction of reactivator and substrate for the binding site of the enzyme. Fraction a_i/a_0 introduces a correction for subtotal inhibition of enzyme in equation (2). A computer program for non-linear regression was used to calculate reactivation parameters. Calculated parameters were k_R, K_R and K_{dis}. Other parameters in the equation were introduced as constants.

The second-order rate constant of reactivation (k_r) was calculated using equation (3):

$$k_r = k_R/K_R \quad (3)$$

Determination of the Dissociation Constant for Enzyme-reactivator Complex

An appropriate volume of 0.01 M solution of the oxime was added to rat brain homogenate (0.5 ml) so as to achieve the desired oxime concentration C_R in the range $5{\cdot}10^{-5}$–$5{\cdot}10^{-2}$ M. Then 0.02 M solution of acetylcholine iodide (2.0 ml) was added, the mixture was adjusted to 25.0 ml with 0.3 M sodium chloride and the rate of the enzyme reaction (v) was immediately determined in the usual manner. The dissociation constant (K_{dis}) was obtained by non-linear regression from the dependence of v on C_R using equation (4):

$$v = v_{max} \times C_s/\left(C_s + K_M \times (1 + C_R/K_{dis})\right) \quad (4)$$

where, in addition to the above-defined symbols, v_{max} denotes the maximum (limiting) rate of the enzymatic reaction, C_s is the substrate concentration and K_M is the Michaelis constant for hydrolysis of acetylcholine by AChE ($1.9{\cdot}10^{-4}$ M).

Statistical Evaluation

Statistical significance was determined by the use of Student's t-test and differences were considered significant when $P < 0.05$. Statistical evaluation was determined with the relevant computer programs.[13]

RESULTS

The ability of tested monopyridinium and bispyridinium oximes to reactivate AChE inhibited by selected nerve agents *in vitro* as characterized by percentage of reactivation and kinetic parameters is summarized in Tables I–IV and Figures 2–5.

The kinetics parameters shown in Table I characterize the ability of all studied oximes to reactivate VX-inhibited AChE *in vitro*. The concentration–reactivation relationship is expressed in Figure 2. The values of the dissociation constant

TABLE I Kinetic parameters of the reactivation of VX-inhibited AChE in rat brain homogenate *in vitro*

Oxime	K_{dis} [μM]	K_R [μM]	k_R [min^{-1}]	k_r [$min^{-1} M^{-1}$]
2-PAM	210	127	0.047	370
Obidoxime	280	562	0.330	587
HI-6	24	130	0.191	1469
K048	228	208	0.174	837

TABLE II Kinetic parameters of the reactivation of tabun-inhibited AChE in rat brain homogenate *in vitro*

Oxime	K_{dis} [μM]	K_R [μM]	k_R [min^{-1}]	k_r [$min^{-1}M^{-1}$]
2-PAM	210	575	0.006	10
Obidoxime	280	3	0.020	6250
HI-6	24	6	0.007	1111
K048	228	93	0.032	348

TABLE IV Kinetic parameters of the reactivation of cyclosarin-inhibited AChE in rat brain homogenate *in vitro*

Oxime	K_{dis} [μM]	K_R [μM]	k_R [min^{-1}]	k_r [$min^{-1}M^{-1}$]
2-PAM	210	12000	0.040	3
Obidoxime	280	-	-	-
HI-6	24	12	0.350	29000
K048	228	-	-	-

(K_{dis}) indicating the affinity of oximes toward the non-inhibited AChE show that the oxime HI-6 has a higher affinity for the intact enzyme than the commonly used obidoxime and pralidoxime as well as the oxime K048. The dissociation constant K_R, which characterizes the affinity of oximes for the inhibited AChE (inhibitor-enzyme complex), indicates that the affinity of HI-6, obidoxime and the new oxime K048 to the enzyme-inhibitor complex is comparable and lower compared to pralidoxime. The differences in the affinity of the studied oximes to intact or inhibited enzyme corresponded to the differences in the second-order rate constant for reactivation of VX-inhibited AChE (k_r). The velocity of HI-6-induced reactivation of VX-inhibited AChE is much higher compared to that for the other oximes studied. According to the results shown in Figure 2, the oximes HI-6, K048 and obidoxime have approximately the same potency to reactivate VX-inhibited AChE at 10^{-4} M, a concentration that should be safe for human use. Obidoxime and the new oxime K048 are able to increase their reactivation potency at 10^{-3} M, nevertheless, this concentration is too high and toxic for human use.

Kinetics parameters in Table II characterize the ability of all studied oximes to reactivate tabun-inhibited AChE *in vitro*. The concentration–reactivation relationship is expressed in Figure 3. While kinetic parameters demonstrate relatively high affinity of obidoxime and HI-6, medium affinity of new oxime K048 and low affinity of pralidoxime toward tabun-inhibited AChE and corresponding values of the second-order rate of reactivation of tabun-inhibited AChE, their potency to reactivate tabun-inhibited AChE is relatively low. According to the results shown in Figure 3, only obidoxime and K048 are able to significantly reactivate tabun-inhibited AChE at 10^{-4} M, while pralidoxime is completely ineffective and the oxime HI-6 is almost ineffective. In addition, the new oxime K048 is able to increase its reactivation potency at higher concentration (10^{-3} M–10^{-2} M), nevertheless, this concentration is probably too high and toxic for human use.

TABLE III Kinetic parameters of the reactivation of sarin-inhibited AChE in rat brain homogenate *in vitro*

Oxime	K_{dis} [μM]	K_R [μM]	k_R [min^{-1}]	k_r [$min^{-1}M^{-1}$]
2-PAM	210	354	0.140	403
Obidoxime	280	781	0.380	486
HI-6	24	9	0.210	22000
K048	228	240	0.0683	284

The ability of all studied oximes to reactivate sarin-inhibited AChE *in vitro* is demonstrated by the kinetic parameters in Table III and by the concentration–reactivation relationship expressed in Figure 4. The values of the dissociation constant K_R indicate that the affinity of HI-6 to the enzyme-inhibitor complex is approximately 20–80 times higher compared to that for the other oximes studied. The differences in the affinity of the studied oximes to intact or inhibited enzyme corresponded to the differences in the second-order rate constant for reactivation of sarin-inhibited AChE (k_r). According to the results shown in Figure 4, the oxime HI-6 is sufficiently effective to reactivate sarin-inhibited AChE in concentrations from 10^{-6} to 10^{-4} M which should be safe for human use. On the other hand, K048, obidoxime and pralidoxime are only able to sufficiently reactivate sarin-inhibited AChE *in vitro* at higher concentration (from 10^{-3} to 10^{-2} M) which would be toxic for human use.

The kinetic parameters shown in Table IV characterize the ability of all studied oximes to reactivate cyclosarin-inhibited AChE *in vitro*. The concentration–reactivation relationship is expressed in Figure 5. The dissociation constant K_R, indicates that the affinity of HI-6 to the enzyme-inhibitor complex is 1000 times higher compared to that for pralidoxime. The differences in the affinity of the studied oximes to intact or inhibited enzyme corresponded to the big differences in the second-order rate constant for reactivation of cyclosarin-inhibited AChE (k_r). The kinetic parameters for obidoxime and K048 could not be measured because of their extremely low potency in reactivating cyclosarin-inhibited AChE. According to the results shown in Figure 5, only the oxime HI-6 is sufficiently effective to reactivate cyclosarin-inhibited AChE in concentrations from 10^{-6} to 10^{-4} M which should be safe for human use. On the other hand, K048, obidoxime as well as pralidoxime are practically ineffective in reactivating cyclosarin-inhibited AChE *in vitro*.

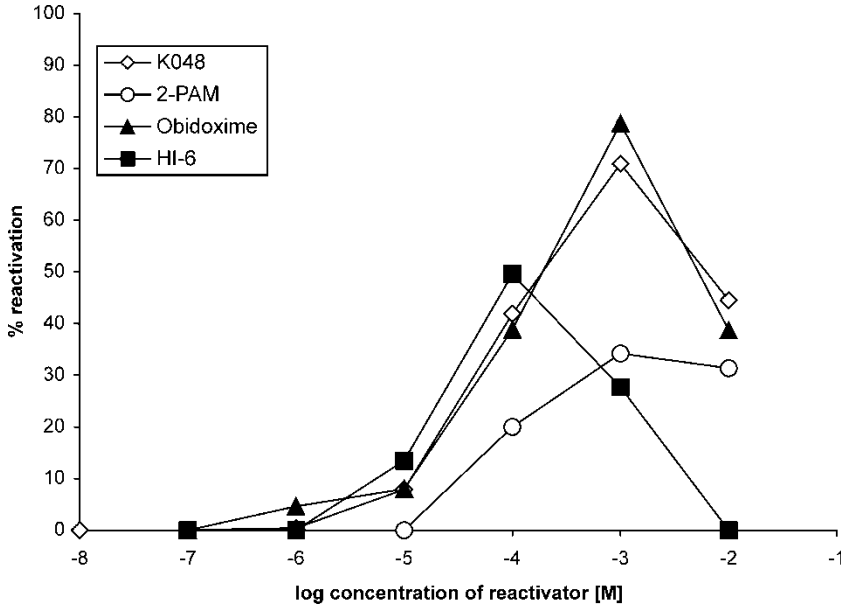

FIGURE 2 Reactivation–concentration relationships of oximes to VX-inhibited AChE–semilogarithmic transformation.

DISCUSSION

The efficacy of AChE reactivators depends on their reactivity and affinity to nerve agent-inhibited enzyme. Their reactivity is derived from the nucleophilic activity of the oxime anion attached to the pyridinium ring.[14] The reactivity of all oximes studied (k_R) is similar because of their similar basic structure. They differ from each other by the number of pyridinium rings (monopyridinium vs bispyridinium oximes) present, position of the oxime group on the pyridinium ring and, in the case of bispyridinium oximes, by the chemical structure of the bridge between both pyridinium rings (K048 vs HI-6 and obidoxime) only.

The affinity of oximes for the intact enzyme, characterized by K_{dis}, and for nerve agent-inhibited enzyme, characterized by K_R, is derived from various physical features (steric compatibility, electrostatic effects, hydrophobic interactions) and from the shape and the size of the whole molecule as well as functional groups.[15] The affinity of reactivators for nerve agent-inhibited AChE is considered to be the most important factor for their reactivating efficacy.

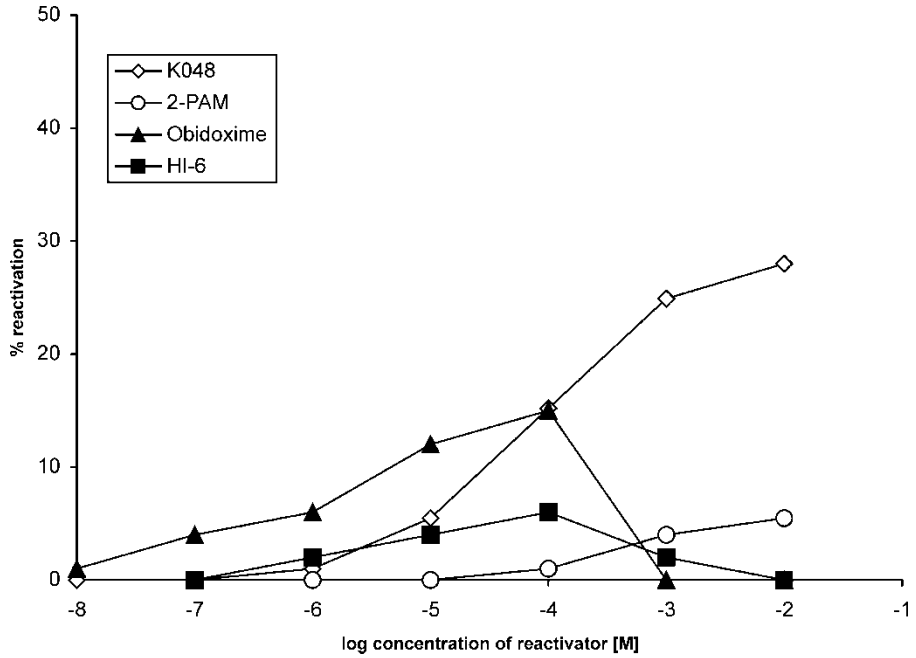

FIGURE 3 Reactivation–concentration relationships of oximes to tabun-inhibited AChE–semilogarithmic transformation.

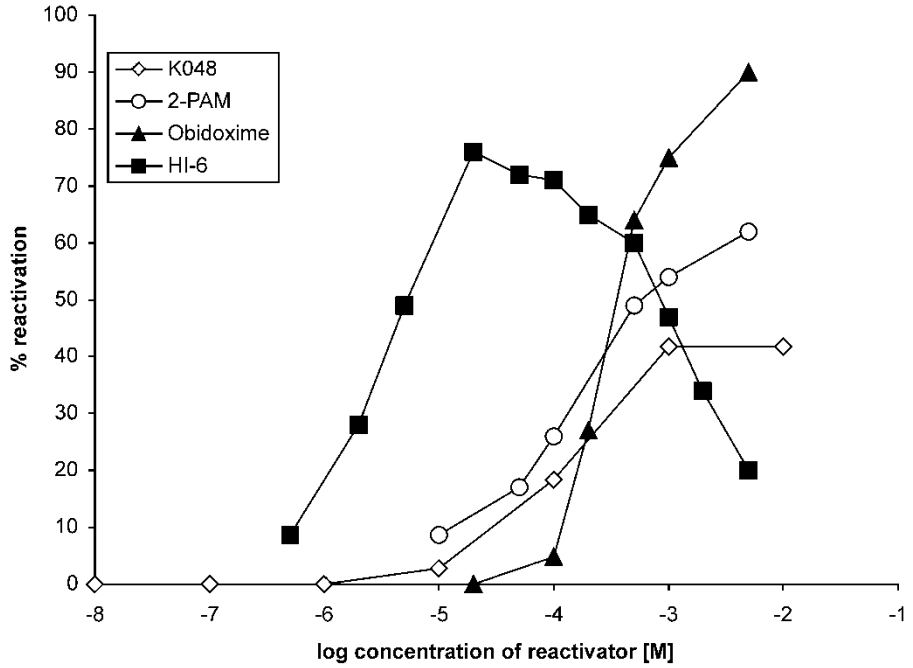

FIGURE 4 Reactivation–concentration relationships of oximes to sarin-inhibited AChE–semilogarithmic transformation.

Our results demonstrate that the strength of reactivator binding to AChE is usually decreased because of reduction of the available space in the cavity of the AChE molecule following enzyme phosphonylation or phosphorylation. While the strength of binding of oximes to AChE is not practically reduced in the case of VX-inhibited AChE, other nerve agents such as sarin and, especially, cyclosarin reduce the strength of binding of oximes to AChE and, thus, make their nucleophilic effects more difficult.[9,11] Despite this fact, the oxime HI-6 seems to be a very good reactivator of sarin or cyclosarin-inhibited AChE *in vitro* because its affinity for sarin or cyclosarin-inhibited AChE is relatively high. On the other hand, currently available oximes (pralidoxime and obidoxime) as well as newly synthesized oxime K048 have significantly lower affinity for sarin or cyclosarin-inhibited AChE and,

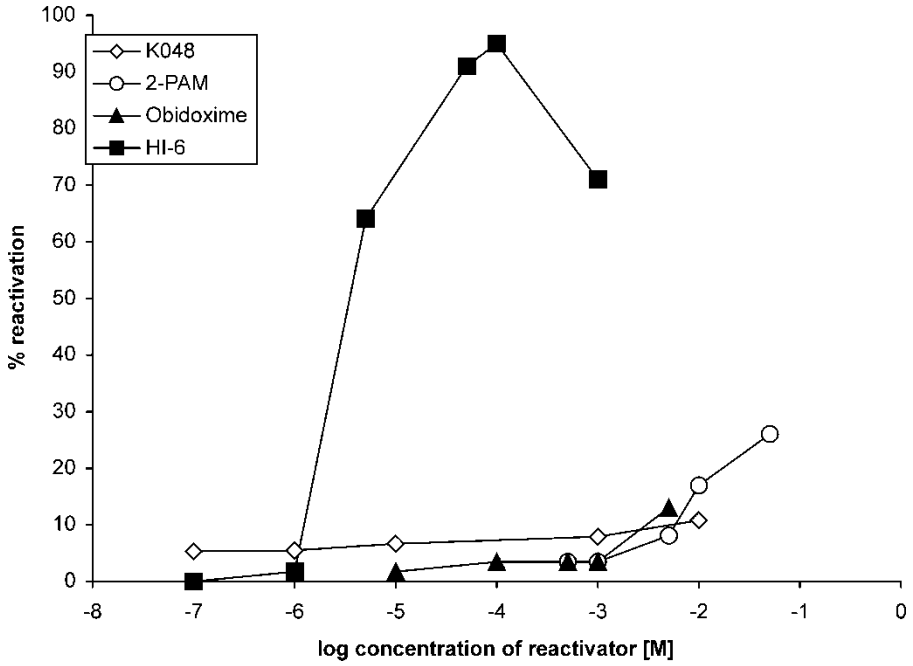

FIGURE 5 Reactivation–concentration relationships of oximes to cyclosarin-inhibited AChE–semilogarithmic transformation.

thus, their reactivating potency, found *in vitro*, is much lower in comparison with HI-6.

The *in vitro* potency of all studied oximes to reactivate tabun-inhibited AChE is completely different from their ability to reactivate VX, sarin or cyclosarin-inhibited AChE. In spite of the relatively high affinity of HI-6 to tabun-inhibited AChE, their potency to reactivate tabun-inhibited AChE is very low. Tabun-inhibited AChE is extraordinarily difficult to reactivate because of the existence of a lone electron pair located on an amidic group that makes the nucleophilic attack of monopyridinium as well as bispyridinium oximes almost impossible.[16–19] The reactivating efficacy of bispyridinium oximes strongly depends on the chemical structure of the bridge connected the two pyridinium rings, the position of the oxime groups and the chemical structure of the substituent situated on the second pyridinium ring.[10] This fact can explain the relatively low efficacy of the oxime HI-6, so efficacious against fluorophosphonates, against tabun because the oxime HI-6 contains an ether bridge and carbamide group instead of the oxime group on the second pyridinium ring. Therefore, newly synthesized oxime K048 that differs from the oxime HI-6 by the structure of the bridge connecting both pyridinium rings (butane instead of ether) and by the position of the oxime group on the pyridinium ring, seems to be a more efficacious reactivator of tabun-inhibited AChE *in vitro* than HI-6. The potency of obidoxime to reactivate tabun-inhibited AChE is also higher compared to HI-6 because of a more suitable positioning of both oxime groups.

Our results confirm that is very difficult to find a broad-spectrum oxime suitable for the antidotal treatment of poisoning by all highly toxic organophosphorus agents.[3,4] The oxime HI-6 so promising against fluorophosphonates such as soman, sarin and cyclosarin[8,9,11] is practically ineffective in reactivating tabun-inhibited AChE.[10] Obidoxime, which is able to reactivate tabun-inhibited AChE, is practically ineffective in reactivating soman or cyclosarin-inhibited AChE.[8,9] Therefore, the newly synthesized bispyridinium oxime K048 which is able to reactivate VX and cyclosarin-inhibited AChE as well as obidoxime but less than HI-6 and also able to reactivate tabun-inhibited AChE more than HI-6 and obidoxime, seems to be, among known oximes, another promising broad-spectrum oxime for antidotal treatment of nerve agent poisoning according to our evaluation of *in vitro* reactivating efficacy.

Acknowledgements

The authors are grateful to Mrs. M. Hrabinová for her skilful technical assistance. The study was supported by a grant from the Ministry of Defense OBVLAJEP20032.

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