Journal of Enzyme Inhibition and Medicinal Chemistry, February 2004 Vol. 19 (1), pp. 39–43

Reactivation of Cyclosarin-inhibited Rat Brain Acetylcholinesterase by Pyridinium–Oximes

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(Received 28 August 2003)

Cyclohexyl methylphosphonofluoridate (cyclosarin, cyclosin, GF) is a highly toxic organophosphate, which is resistant to conventional oxime therapy. To gain insight into the reactivation kinetics, rat brain acetylcholinesterase (AChE) was inhibited *in vitro* by cyclosarin (pH 8.0, 25°C) and reactivated with 22 different pyridinium–oximes. Three compounds were shown to be superior to the other oximes: 4-carbamoyl-4′-[(hydroxyimino)-methyl]-1,1′-(oxydimethylene)dipyridin-1-ium dichloride (HS-6), 4′-carbamoyl-2-[(hydroxyimino)methyl]-1,1′-(oxydimethylene)dipyridin-1-ium dichloride (HI-6), and 4′-carbamoyl-2-[(hydroxyimino)-methyl]-1,1′-(but-2-ene-1,4-diyl)dipyridin-1-ium dichloride (BI-6).

Keywords: acetylcholinesterase; oxime; reactivation; cyclosarin; *in vitro*

INTRODUCTION

The organophosphate nerve agents tabun (GA), sarin (GB), soman (GD), and cyclosarin (GF) rank among the most toxic chemical warfare agents known.[1,2] They are also called the G-series nerve agents, because German scientists first synthesized them, starting with tabun (GA) in 1936. Sarin (GB) was discovered in 1938, followed by soman (GD) in 1944 and finally the less known cyclosarin (GF) in 1949.[3] The V-series nerve agent VX was developed subsequently.[4] The nerve agents, sarin, soman, and VX are organophosphorus esters that form a major part of the total agent volume in the stockpile of unitary chemical ammunitions. All these organophosphates are very poisonous and their toxic effect is based on the inhibition of acetylcholinesterase (AChE, EC 3.1.1.7), the enzyme vital for cholinergic transmission in the central and peripheral nervous system.[5,6] Organophosphate-inhibited AChE can be reactivated by powerful nucleophiles such as pyridinium–aldoximes.[7,8] GF, cyclohexyl methylphosphonofluoridate, is the chemical warfare agent that may have been used in the Gulf War operations.[9] This, a highly toxic but so far little explored organophosphate, was shown to be resistant to conventional oxime therapy.[10–14] The aim of this work is to compare the *in vitro* reactivation ability of selected pyridinium–oximes in a model of rat brain AChE inhibited by the GF.

MATERIALS AND METHODS

Chemicals

Oxime HS-6 (**21**) was obtained from Dr Stojiljkovic (Serbia and Montenegro). Pralidoxime, and trimedoxime were purchased from Léčiva (Czech Rep) and obidoxime from Merck (Germany). All other oxime reactivators of organophosphate-inhibited acetylcholinesterase used in this study were prepared earlier in our laboratory.[15–18] Their chemical structures are given in Table 1. They are quaternary pyridinium–oximes with one or two (hydroxyimino)methylgroups. Cyclosarin was obtained from a military facility (VOZ 072) Zemianské Kostolany, Slovak Republic, in 98% purity. All other chemicals were obtained from commercial sources and were of reagent grade.

In Vitro Experiments

The reactivation ability of the tested oximes has been assayed *in vitro* on a model of AChE inhibited by cyclosarin using a standard reactivation test.[19]

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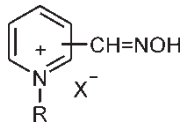
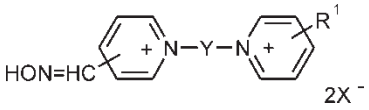
ISSN 1475-6366 print/ISSN 1475-6374 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/1475636031000163850

TABLE I Reactivation effectivity of the tested oximes [10^{-3} M] on GF-inhibited AChE



No.	Code	Position of –CH=NOH	R	Percentage of reactivation (%)
1	2-PAM	2	CH_3	2.5
2	TO 020	2	CH_2Ph	0
3	4-PAM	4	CH_3	3
4	TO 063	4	$(CH_2)_5CH_3$	0
5	TO 061	4	CH_2Ph	0



No.	Code	Position of –CH=NOH	R^1	Y	Percentage of reactivation (%)
6	TO 091	4	4–CH=NOH	$(CH_2)_{10}$	0
7	TO 090	4	4–CH=NOH	$(CH_2)_8$	0
8	TO 033	4	4–CH=NOH	$(CH_2)_6$	0
9	TO 047	4	4–CH=NOH	$(CH_2)_5$	0
10	TO 046	4	4–CH=NOH	$(CH_2)_4$	0
11	Trimedoxime	4	4–CH=NOH	$(CH_2)_3$	0
12	TO 029	4	4–CH=NOH	$(CH_2)_2$	0
13	MMC	4	4–CH=NOH	$(CH_2)_1$	32
14	TO 057	4	4–CH=NOH	$CH_2CH_2SO_2CH_2CH_2$	8
15	TO 058	4	4–CH=NOH	$CH_2CH_2S^+(CH_3)H_2CH_2$	0
16	TO 205	4	4–CH=NOH	$CH_2CH = CH_2CH_2$	0
17	Obidoxime	4	4–CH=NOH	CH_2OCH_2	0
18	TO 052	4	4–CH=NOH	CH_2COCH_2	3
19	BI-6	2	4–$CONH_2$	$CH_2CH = CH_2CH_2$	57
20	HI-6	2	4–$CONH_2$	CH_2OCH_2	58
21	HS-6	4	4–$CONH_2$	CH_2OCH_2	70
22	TO 092	4	–H	$(CH_2)_3$	0

As a source of AChE, a homogenate of rat brains (rats of Wistar strain, 200–240 g) without sex preference was used. The animals were killed under narcosis by cutting the carotids, the brains were removed, rinsed in physiological saline and homogenized in an Ultra-Turrax (Germany) homogenizer in distilled water to make a 10% homogenate.

We have used rat brain homogenate as a source of the enzyme for the following reasons:

(i) Rat brain homogenate has been using in our experiments for many years.[20,21] Therefore, we can compare our new results with previous results. (ii) *In vitro* experiments are followed by *in vivo* experiments using experimental animals and to compare *in vitro* with *in vivo* results, the same source of AChE should be used.[22,23] (iii) Data from animal experiments are indispensable for the prospective licensing of an oxime as a drug candidate by regulatory authorities.[24]

The AChE homogenate (0.5 ml) was mixed with 0.5 ml of 1 μM GF and incubated at 25°C for 30 min. Then, 2.5 ml of 3 M NaCl was added and the mixture adjusted with distilled water to a final volume of 23 ml. Then, 2 ml of 0.02 M acetylcholine bromide was added and the enzyme activity was assayed titrimetrically at pH 8.0 and 25°C using an Autotitrator RTS 822 (Radiometer, Copenhagen). The activities of intact (a_o) and GF-inhibited (a_i) AChE were determined. When GF-inhibited AChE was incubated for 10 min with a solution of an oxime reactivator, the activity of reactivated AChE (a_r) was obtained. The activity values a_0, a_i and a_r, were calculated from the initial slopes of the titration curves. Each value is the arithmetic mean of two independent measurements.

The kinetics of the reactivation process may be represented by the scheme:[18]

$$EI + R \overset{K_R}{\rightleftharpoons} EIR \xrightarrow{k_R} E + P \qquad (1)$$

where EI is the organophosphate-inhibited enzyme, R is the reactivator, E is the reactivated enzyme, EIR is the intermediate complex, and P is the product, usually phosphomylated unstable oxime. K_R and k_R are the dissociation constants and the rate constants for decomposition of the intermediate complex, respectively.[25] For all the oximes whose reactivation ability was screened, the percentage of reactivation (%R) was calculated from the equation,[20]

$$\%R = \left[1 - \frac{a_0 - a_r}{a_0 - a_i}\right] \cdot 100 \qquad (2)$$

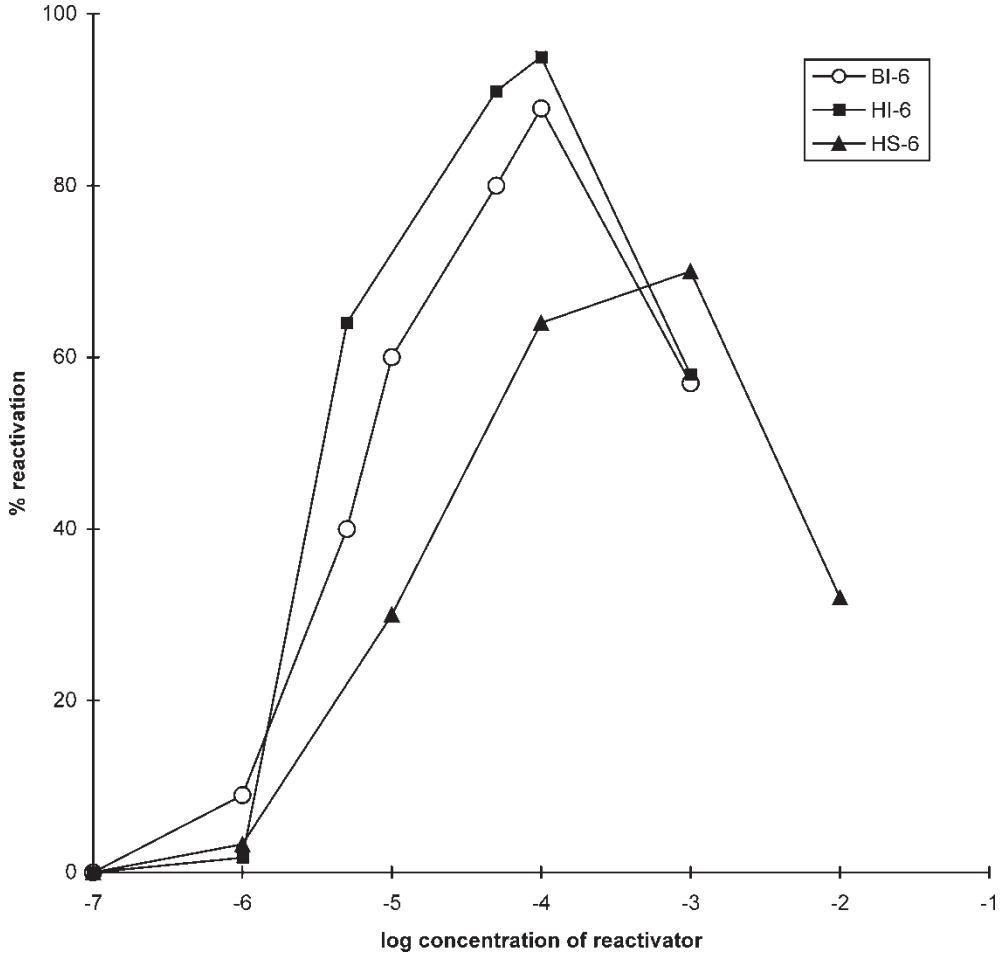


FIGURE 1 Concentration–reactivation relationship of pyridinium–oximes in reactivation of GF-inhibited AChE.

The percentage of reactivation of effective reactivators was measured in the range of oxime concentrations from 10^{-7} to 10^{-2} M (Figure 1). Kinetic constants of reactivation were obtained from the equation,

$$k_{app} = k_0 + k_R \frac{c_R}{K_R + c_R}. \quad (3)$$

where,

$$k_{app} = -\frac{1}{t} \ln \frac{a_0 - a_r}{a_0 - a_i} \quad (4)$$

The dissociation constant of the oxime–reactivator complex with inactive, i.e. non-phospomylated acetylcholinesterase (K_R), was obtained from the equation,

$$a_i = \frac{a_0 c_s}{c_s + K_M\left(1 + \frac{c_R}{K_R}\right)} \quad (5)$$

by non-linear regression analysis of a_i on c_R, where a_o is enzymatic activity of non-GF-inhibited AChE, a_i is enzyme activity of GF-inhibited AChE, c_s is concentration of substrate, c_R is concentration of oxime and K_M is Michaelis constant for acetylcholine as substrate, equal to 0.19 mM.[20] Second-order rate constants of reactivation (k_r), which represent overall reactivation ability, were calculated from the equation,[18]

$$k_r = k_R / K_R \quad (6)$$

RESULTS

The results obtained at an oxime concentration of 1 mM are summarized in Table I.

Only three pyridinium–oximes (**19**, **20**, **21**) exhibited more than 50% reactivation. The other oximes tested showed lower or no reactivation ability (Table 1). The most effective reactivator of GF-inhibited AChE at the 10^{-3} M concentration is HS-6 (**21**), while HI-6 (**20**) and BI-6 (**19**) are slightly less effective. These three compounds were further investigated and all their kinetic constants obtained are summarized in Table II.

The first constant — K_{DIS} indicates affinity of oximes toward the non-inhibited AChE. Oxime HS-6

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

Compound	K_{DIS} (μM)	K_R (μM)	k_R (min^{-1})	k_r ($M^{-1} min^{-1}$)	pK_a
HS-6	363	44	0.16	3700	8.00
BI-6	42	20	0.25	13000	7.63
HI-6	24	12	0.35	29000	7.50

K_{DIS}, dissociation constant of the enzyme-reactivator cpmplex; 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; pK_a, dissociation constant of reactivator.

has the lowest affinity (the highest value of the K_{DIS}) to the intact enzyme in comparison with the other tested oximes. Dissociation constant K_R is the highest for the oxime HS-6, too. Oximes BI-6 and HI-6 have comparable values of both dissociation constants. The oxime HS-6 has the lowest rate constant for the breakdown of the intermediate complex — k_R, as well as the lowest second order rate constant of reactivation — k_r.

The ability of all three studied oximes to reactivate GF-inhibited AChE is shown in Figure 1. The best reactivator of GF-inhibited AChE seems to be H-oxime HI-6. 10^{-4} M concentration of HI-6 is sufficient to give 95% reactivation of GF-inhibited AChE. Oxime BI-6 was able to reactivate GF-inhibited AChE to the extent of 89% and oxime HS-6 only 64% at the same concentration. Oxime HS-6 was even able to reactivate GF-inhibited AChE to the extent of 70% reactivation but the concentration of the oxime HS-6, necessary for this to occur [10^{-3} M] is too high for human use.

DISCUSSION

An ideal reactivator of GF-inhibited AChE should have a value of K_R from 10–100 fold lower than the K_m of the native substrate (K_m for AChE + acetylcholine iodide is 200 μM) and the value of K_{DIS} from 10–100 times higher than K_m.[22,23] Thus, the affinity of the AChE reactivator to the inhibited enzyme should be higher than the affinity to the reactivated, respectively, intact enzyme. This specification favours oxime HS-6 more than the oximes HI-6 and BI-6.

The differences in the values of rate constant (k_R and k_r) and dissociation constants (K_{DIS} and K_R) depend on many factors — position and number of the oxime group, number of quaternary nitrogens, shape of the linking chain, presence of the carbamoyl moiety and p*K*a of the oxime group.[17,26,27]

The number of aldoxime groups is not so important. On the other hand, the position of the oxime group in the pyridinium ring can play an important role in the reactivation efficacy of oximes. The potency of reactivators to reactivate GF-inhibited AChE with the oxime group at position-4 is lower compared to reactivators with the oxime group at the position-2, i.e. 2- (**19**, **20**) > 4- (**21**).[28]

A characteristic feature of the AChE-reactivators is two positive charges on two pyridine nitrogens linked with the equivalent tri- or tetra-carbon chain.[28–30] The chain linking the two quaternary nitrogens in pyridinium–oximes **19**, **20**, **21** exerts a great effect on the reactivation kinetics, although this part of the oxime reactivator molecule does not play any role in the dephosphorylation process. Our findings suggest that the connecting chain is a major factor in influencing oxime access and reactivation rates.

Our results also confirm the rule that an important part of effective reactivators is the carbamoyl group in position-4 of one pyridinium moiety.[31]

The rate constant of reactivation of compounds **19**, **20** and **21** is in good correlation with pK_a values, hence it is evident that the reactivation rate is dependent on the extent of oxime-group ionization.[20]

In conclusion, our results confirm that our screening method can select potential AChE-reactivators. Nevertheless, to evaluate the reactivation potency of the potential AChE-reactivators more precisely, their reactivation ability in the range of 10^{-7}–10^{-2} M needs to be measured.

Acknowledgements

The authors are grateful to Mrs. I. Ježková and Mrs. M. Hrabinová for technical assistance. The study was supported by a grant from the Ministry of Defense, No. 9079101301 - Czech Republic.

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