

ORIGINAL ARTICLE

Could oxime HI-6 really be considered as “broad-spectrum” antidote?

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Summary

The broad-spectrum reactivator is a valuable oxime able to reactivate acetylcholinesterase (AChE) inhibited by nerve agents and pesticides. At present there are many AChE reactivators (oximes) which are suitable candidates as broad-spectrum reactivators and among them is the oxime HI-6, highly enough thought of to have been recommended by many armies for use as a universal antidote. In this study, we wanted to establish whether the designation “broad-spectrum” is an accurate description or if there are some lacks in reactivation of nerve agents or pesticides. For this purpose, the general *in vitro* test for the evaluation of AChE reactivators was used. Tabun, sarin, cyclosarin, soman, VX agent, Russian VX were used as nerve agents for testing, and chlorpyrifos, paraoxon, methyl-chlorpyrifos and dichlorvos (DDVP) were used as typical examples of organophosphorus pesticides. The results obtained showed that oxime HI-6 did not reactivate tabun- and DDVP-inhibited AChE, and, in the case of the other pesticides, only a high dose of oxime HI-6 was able to reactivate pesticide-inhibited AChE.

Key words: acetylcholinesterase; reactivator; oxime; nerve agent; pesticide; HI-6; broad-spectrum

INTRODUCTION

After the Tokyo sarin subway attack, it was clear that there exists potential for the misuse of chemical

warfare agents, especially nerve agents, as the weapons of terrorist groups or individuals (Tu 2000). The preparation of appropriate antidotes has therefore become a priority. The current antidotes for nerve agent poisoning, are anticholinergics, acetylcholinesterase (AChE) reactivators and anticonvulsants. Atropine is currently the best anticholinergic drug, commonly used in antidote formulations. Diazepam or its pro-drug avizafone are the best candidates for treatment of convulsions. However in the case of the AChE reactivator, there are five commercially available oximes – pralidoxime (2-PAM; P2S), trimedoxime (TMB-4), obidoxime (toxogonin; Lüh-7), methoxime (MMC4; MMB-4)

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and oxime HI-6 (Kuča and Kassa 2004a, b; Žďárová Karasová et al. 2009). According to the literature, pralidoxime, although it is still used worldwide for the treatment of pesticide poisoning, seems to be already old-fashioned and has been replaced by obidoxime (Petroianu et al. 2007a, b, c). Obidoxime is generally used for the treatment of pesticide poisoning (Thiermann et al. 1999), however its use as an antidote against nerve agents is limited, especially in the case of cyclosarin and soman poisoning (Kassa et al. 2007a). Trimedoxime has a similar reactivation potency as obidoxime, it is recommended more as a treatment for tabun-poisoning (Kassa et al. 2007a). For these reasons, two other reactivators – methoxime and HI-6 are nowadays being thoroughly investigated throughout the world. This research can be divided into two parts – methoxime is under investigation in the US (Singh et al. 2007) and HI-6 is the subject of research in many other countries such as England, Germany, France, Canada, Czech Republic, etc. (Fig. 1). If the efficacy of both these oximes is compared by perusal of published articles, HI-6 has many more advantages than methoxime, and because of this, HI-6 is at present number one among the oxime reactivators (Kassa et al. 2007b, Kuča et al. 2007).

With all the above in mind, we wanted to establish if oxime HI-6 could be considered as a broad-spectrum reactivator, able to sufficiently reactivate AChE inhibited by all kinds of nerve agents and pesticides. Such a property is very important in view of the possible misuse of an unidentified nerve agent (i.e. where appropriate detectors of nerve agents are not available). Currently, a reactivation kit consisting of several oximes would be used to find the most active oxime which would then be recommended as an effective treatment. A so called broad-spectrum reactivator could save time in the identification of an antidote and intoxication could be treated immediately.

To evaluate the broad-spectrum reactivation potency of oxime HI-6, a broad range of nerve agents have to be tested. We therefore selected for our study six nerve agents (tabun, sarin, cyclosarin, soman, VX agent, Russian VX) and four organophosphorus pesticides [chlorpyrifos, paraoxon, methyl-chlorpyrifos and dichlorvos (DDVP)] (Fig. 2).

MATERIAL AND METHODS

Oxime HI-6 was prepared in our department according to the synthesis described by Kuča et al. (2008). Its structure is shown in Fig. 1. The purity of

this reactivator was detected using the TLC technique, the HPLC technique and NMR (Jun et al. 2007, 2008). All nerve agents were obtained from the Military Facility Brno (95% purity and higher). Pesticides were obtained from Sigma-Aldrich as analytical standards. All other chemicals used were of reagent grade (Sigma-Aldrich, Czech Republic).

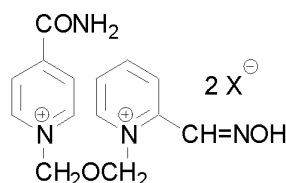


Fig. 1. Structure of the oxime HI-6.

Rat brains were chosen as the appropriate source of cholinesterases. Their preparation was as follows: Lightly ether-narcotized animals (the narcosis used did not influence cholinesterase activity – Novotný et al. 2009) were killed by bleeding from a carotid artery and then the brains were removed, washed with saline and homogenized using an Ultra-Turrax homogenizer in distilled water to make a 10% homogenate (w/v). The animals used in this study were handled under the supervision of the Ethics Committee of the Faculty of Military Medical Academy in Hradec Králové, Czech Republic.

In vitro testing of oxime HI-6 has been described in detail already earlier by Kuča and Cabal (2005). Briefly, the 10% rat brain homogenate in distilled water was used as a source of AChE. The brain homogenate (0.5 ml) was mixed with 20 µl of the isopropanol solution of an appropriate acetylcholinesterase inhibitor and distilled water (0.5 ml). The mixture was incubated at 25 °C for 30 minutes to achieve 95% inhibition of AChE (in case of somam – only five-minute incubation was used because of aging). The mixture was filled in an assay vessel to the volume 23 ml with distilled water, and sodium chloride (3M; 2.5 ml) was added. Finally, 2 ml of acetylcholine iodide (0.02M; substrate for enzymatic reaction) was added. The enzyme activity (analyzed by potentiometric titration of decomposed acetylcholine iodide) was measured at pH 7.6 and 25 °C using an autotitrator RTS 822 (Radiometer, Denmark).

The same procedure was repeated with the enzyme, which was incubated for 30 min with the appropriate AChE inhibitor and further treated for 10 min with an aqueous solution of the reactivator (0.2 ml – replacing same amount of distilled water). The activity of the intact AChE (a_0), inhibited AChE (a_i) and reactivated AChE (a_r) were calculated from

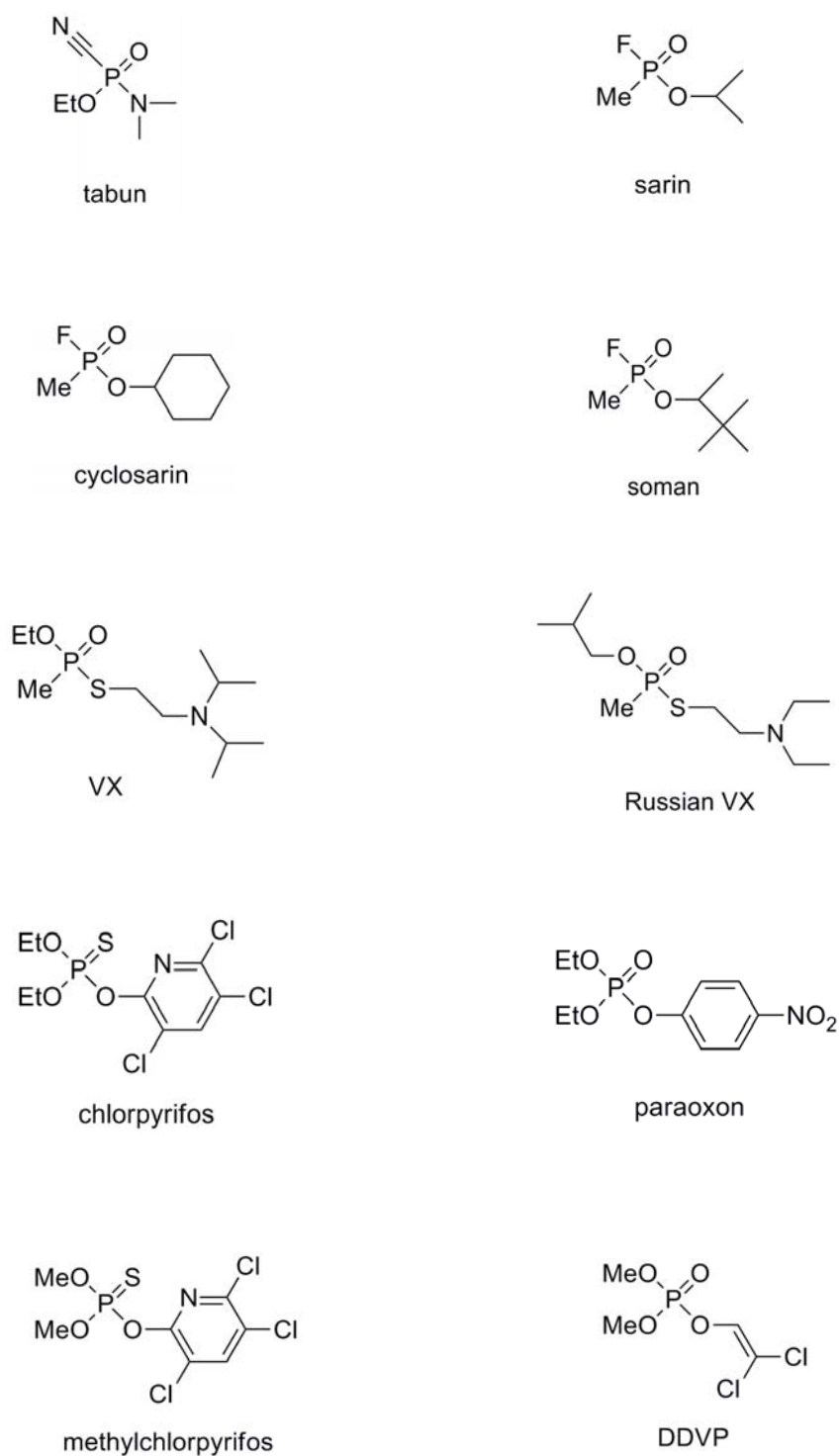


Fig. 2. Structures of used nerve agents and pesticides.

the amount of NaOH solution (0.01 M) versus time; NaOH reacted with acetic acid released from decomposed acetylcholine iodide. The percentage of reactivation (%) was calculated from the measured data according to the formula:

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

RESULTS

All the results obtained are summarized in Table 1 and for better visualization also in Fig. 3. Oxime HI-6 was able to reactivate AChE inhibited by all nerve agents and pesticides. However, the reactivation percentage ranged from 2% to 71% depending on the nerve agent or pesticide used and depending on the concentration of the oxime. As is known from the literature, 10% of the reactivation potency is enough

to save the life of an intoxicated organism (Bajgar et al. 2007), so that reactivation values under this limit cannot be considered satisfactory, and therefore, we can say that the oxime is not sufficient for the reactivation of AChE inhibited by a given inhibitor.

Given this limitation, oxime HI-6 is not able to reactivate tabun and DDVP-inhibited AChE. In all other cases it exceeded 10% reactivation.

The concentration of the oxime is another factor to be considered as influencing the reactivation process in the living organism. High oxime concentrations are not allowed because of the risk of overdosing (Bartošová et al. 2006). Overdosing of AChE reactivators has, unfortunately, the same effect as the AChE inhibitors (Pohanka et al. 2007).

From this point of view, reactivation potency at low oxime concentrations should be considered as more likely. Therefore, our attention should be directed on the concentration 10^{-5} M. In this case, also paraoxon- and methyl-chlorpyrifos-inhibited AChE are not reactivated by oxime HI-6.

Table 1. Reactivation of nerve agents- and pesticide-inhibited AChE using oxime HI-6.

Nerve agent	Reactivation [%] (10^{-5} M)	SD	Reactivation [%] (10^{-3} M)	SD
Tabun	4	0	2	0
Sarin	49	6	47	4
Cyclosarin	28	3	52	6
Soman*	16	2	23	3
VX	14	1	28	3
Russian VX	53	7	42	5
Chlorpyrifos	11	1	20	2
Paraoxon	0	0	35	5
Methyl-chlorpyrifos	0	0	13	2
DDVP	2	0	3	0

* only 5 min inhibition because of aging

DISCUSSION

Many scientific departments have in recent times been focused on the synthesis of promising new oximes with increased reactivation potency in the hope of discovering broad-spectrum reactivators. During last five years, more than thirty articles have been published on the topic of this synthesis (Chennamaneni et al. 2005, Kim et al. 2005, 2006, Pícha et al. 2005, Musílek et al. 2006a, b, 2007a, b, Oh et al. 2006, 2008, Ohta et al. 2006, Yang et al. 2007). Unfortunately, none has achieved the

preparation of the so-called broad-spectrum oxime. As a result of this study, oxime HI-6 also cannot be considered as a broad-spectrum compound. In the case of pesticides, HI-6 is almost ineffective (Petroianu et al. 2006a, b, Lorke et al. 2007, 2008a, b, 2009). According to the data in the literature, obidoxime clinically used exerts more promising results in this field (Bond et al. 2008). Moreover, a new candidate for this purpose is now under investigation – oxime K027 (Petroianu et al. 2006a, b, 2007a, b, c, Lorke et al. 2007, 2008a, b, 2009, Tekes et al. 2006, Gyenge et al. 2007, Nurulain et al. 2009).

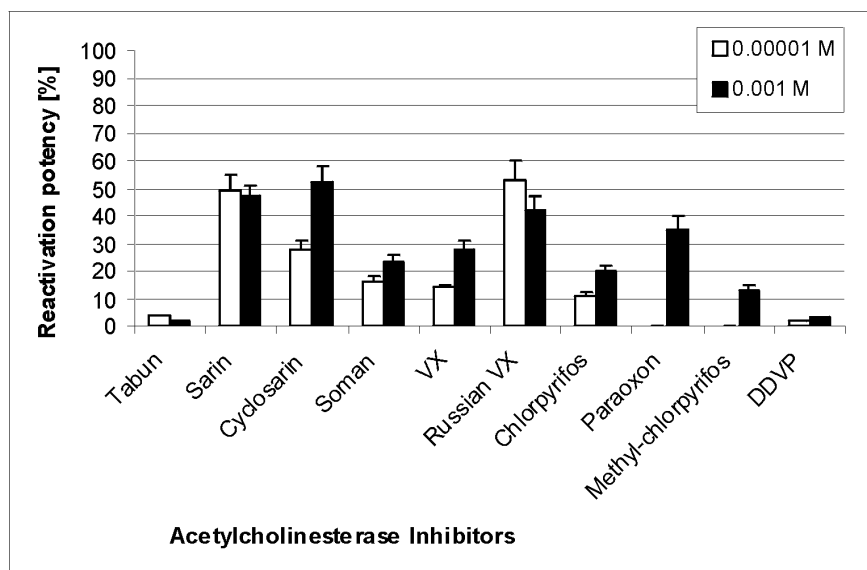


Fig. 3. Efficacy of oxime HI-6 in two concentrations (10^{-3} M and 10^{-5} M) in reactivation of different AChE inhibitors (three independent measurements).

From the point of view of reactivation of nerve agents, the reactivation efficacy of the oxime HI-6 is more promising, except in the case of tabun-inhibited AChE (Kassa et al. 2006). However, Hamilton with his co-workers established in 1989 that HI-6 could influence receptors, and thus, it saved the lives of intoxicated monkeys although no reactivation occurs (Hamilton and Lundy 1989). These data are in conflict with the data obtained on rats, where HI-6 had no benefit (Kassa et al. 2006). This discrepancy could have been caused by the species differences which are well known (Kuča et al. 2005, Wiesner et al. 2007). Thus, HI-6 could be considered as a universal reactivator for the treatment of nerve agent intoxication, with the exception of tabun. If the data obtained for HI-6 are compared with data published in earlier literature for oxime HLö-7 (the reactivator developed and tested as a replacement for HI-6), the results are very similar. Therefore, consideration of HLö-7 as HI-6 replacement is not necessary (Kuča et al. 2006).

According to the results obtained, HI-6 could be recommended as a broad-spectrum reactivator for nerve agents among the presently available oximes. On the other hand, HI-6 is not a good candidate for the treatment of pesticide intoxication. In this case, new candidates should be considered to replace the relatively toxic obidoxime and trimedoxime or pralidoxime.

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REFERENCES

- Bajgar J, Fusek J, Kuča K, Bartošová L, Jun D: Treatment of organophosphate intoxication using cholinesterase reactivators: facts and fiction. *Mini Rev Med Chem* 7:461–466, 2007.
- Bartošová L, Kuča K, Kunešová G, Jun D: The acute toxicity of acetylcholinesterase reactivators in mice in relation to their structure. *Neurotox Res* 9:291–296, 2006.
- Bond GR, Pieche S, Sonicki Z, Gamaluddin H, El Guindi M, Sakr M, El Seddawy A, Abouzaid M, Youssef A: Pediatric Insecticide Study Group. A clinical decision aid for triage of children younger than 5 years and with organophosphate or carbamate insecticide exposure in developing countries. *Ann Emerg Med* 52:617–622, 2008.
- Chennamaneni SR, Vobalaboina V, Garlapati A: Quaternary salts of 4,3' and 4,4'-bis-pyridinium monooximes: Synthesis and biological activity. *Bioorg Med Chem Lett* 15:3076–3080, 2005.
- Gyenge M, Kalasz H, Petroianu G, Laufer R, Kuča K,

- Tekes K: Measurement of K-27, an oxime-type cholinesterase reactivator by high performance liquid chromatography with electrochemical detection from different biological samples. *J Chromatogr A* 1161:146–151, 2007.
- Hamilton MG, Lundy PM: HI-6 therapy of soman and tabun poisoning in primates and rodents. *Arch Toxicol* 63:144–149, 1989.
- Jun D, Stodůlka P, Kuča K, Kolečkář V, Doležal B, Šimon P, Veverka M: HPLC analysis of HI-6 dichloride and dimethanesulfonate – antidotes against nerve agents and organophosphorus pesticides. *Anal Lett* 40:2783–2787, 2007.
- Jun D, Stodůlka P, Kuča K, Kolečkář V, Doležal B, Šimon P, Veverka M: TLC analysis of intermediates arising during the preparation of oxime HI-6 dimethanesulfonate. *J Chromatogr Sci* 46:316–319, 2008.
- Kassa J, Kuča K, Cabal J, Paar M: A comparison of the efficacy of new asymmetric bispyridinium oximes (K027, K048) with currently available oximes against tabun by *in vitro* and *in vivo* methods. *J Toxicol Environ Health A* 69:1875–1882, 2006.
- Kassa J, Kuča K, Bartošová L, Kunešová G: The development of new structural analogues of oximes for the antidotal treatment of poisoning by nerve agents and the comparison of their reactivating and therapeutic efficacy with currently available oximes. *Curr Org Chem* 11:267–283, 2007a.
- Kassa J, Jun D, Kuča K, Bajgar J: A comparison of reactivating and therapeutic efficacy of two salts of the oxime HI-6 against tabun, soman and cyclosarin in rats. *Basic Clin Pharmacol Toxicol* 101:328–332, 2007b.
- Kim TH, Kuča K, Jun D, Jung YS: Design and synthesis of new bis-pyridinium oximes as cyclosarin-inhibited acetylcholinesterase reactivators. *Bioorg Med Chem Lett* 15:2914–2917, 2005.
- Kim TH, Oh KA, Park NJ, Lim YJ, Yum EK, Jung YS: Reactivation study of pyridinium oximes for acetylcholinesterases inhibited by paraoxon or DFP. *J Appl Biomed* 4:67–72, 2006.
- Kuča K, Cabal J, Kassa J: *In vitro* reactivation of sarin-inhibited brain acetylcholinesterase from various species by various oximes. *J Enzyme Inhib Med Chem* 20:227–232, 2005.
- Kuča K, Cabal J: Evaluation of newly synthesized reactivators of the brain cholinesterase inhibited by sarin-nerve agent. *Toxicol Mech Methods* 15:247–252, 2005.
- Kuča K, Kassa J: *In vitro* reactivation of acetylcholinesterase using of the oxime K027. *Vet Hum Toxicol* 46:15–18, 2004a.
- Kuča K, Kassa J: Oximes-induced reactivation of rat brain acetylcholinesterase inhibited by VX agent. *Hum Exp Toxicol* 23:167–171, 2004b.
- Kuča K, Cabal J, Kassa J, Jun D, Hrabínová M: *In vitro* potency of H oximes (HI-6, HLö-7), the oxime BI-6, and currently used oximes (pralidoxime, obidoxime, trimedoxime) to reactivate nerve agent-inhibited rat brain acetylcholinesterase. *J Toxicol Environ Health A* 69:1431–1440, 2006.
- Kuča K, Musílek K, Stodůlka P, Marek J, Hanušová P, Jun D, Hrabínová M, Kassa J, Doležal B: Twelve different HI-6 salts and their potency to reactivate cyclosarin inhibited AChE *in vitro*. *Lett Drug Des Discov* 4:510–512, 2007.
- Kuča K, Stodůlka P, Hrabínová M, Hanušová P, Jun D, Doležal B: Convenient preparation of the oxime HI-6 (dichloride and DMS) – antidote against nerve agents. *Def Sci J* 58:399–404, 2008.
- Lorke DE, Hasan MY, Nurulain SM, Sheen R, Kuča K, Petroianu GA: Entry of two new asymmetric bispyridinium oximes (K-27 and K-48) into the brain: comparison with obidoxime. *J Appl Toxicol* 27:482–490, 2007.
- Lorke DE, Nurulain SM, Hasan MY, Kuča K, Musílek K, Petroianu GA: Eight new bispyridinium oximes in comparison with the conventional oximes pralidoxime and obidoxime: *In vivo* efficacy to protect from diisopropylfluorophosphate (DFP) toxicity. *J Appl Toxicol* 28:920–928, 2008a.
- Lorke DE, Hasan MY, Arafat K, Kuča K, Musílek K, Schmitt A, Petroianu GA: *In vitro* oxime protection of human red blood cell acetylcholinesterase inhibited by diisopropyl-fluorophosphate. *J Appl Toxicol* 28:422–429, 2008b.
- Lorke DE, Hasan MY, Nurulain SM, Kuča K, Schmitt A, Petroianu GA: Efficacy of two new asymmetric bispyridinium oximes (K-27 and K - 48) in rats exposed to diisopropylfluorophosphate: comparison with pralidoxime, obidoxime, trimedoxime, methoxime, and HI-6. *Toxicol Mech Methods* 19:327–333, 2009.
- Musílek K, Holas O, Hambálek J, Kuča K, Jun D, Dohnal V, Doležal M: Synthesis of bispyridinium compounds bearing propane linker and evaluation of their reactivation activity against tabun- and paraoxon-inhibited acetyl-cholinesterase. *Lett Org Chem* 3:831–835, 2006a.
- Musílek K, Lipka L, Račáková V, Kuča K, Jun D, Dohnal V, Doležal V: New methods in synthesis of acetylcholinesterase reactivators and evaluation

- of their potency to reactivate cyclosarin-inhibited AChE. *Chem Pap* 60:48–51, 2006b.
- Musílek K, Jun D, Cabal J, Kassa J, Gunn-Moore F, Kuča K: Design of a Potent Reactivator of Tabun-Inhibited Acetylcholinesterase – Synthesis and evaluation of (*E*)-1-(4-carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide (K203). *J Med Chem* 50:5514–5518, 2007a.
- Musílek K, Kuča K, Jun D, Doležal M: Progress in synthesis of new acetylcholinesterase reactivators during the period 1990–2004. *Curr Org Chem* 11:229–238, 2007b.
- Novotný L, Karasová J, Kuča K, Bajgar J, Misík J: Influence of different wals of euthanasia on the activity of cholinesterases in the rat. *J Appl Biomed* 7:133–136, 2009.
- Nurulain SM, Lorke DE, Hasan MY, Shafiullah M, Kuča K, Musílek K, Petroianu GA: Efficacy of eight experimental bispyridinium oximes against paraoxon-induced mortality: comparison with the conventional oximes pralidoxime and obidoxime. *Neurotox Res* 16:60–67, 2009.
- Oh KA, Yang GY, Jun D, Kuča K, Jung YS: Bis-pyridiumaldoxime reactivators connected with $\text{CH}_2\text{O}(\text{CH}_2)_n\text{OCH}_2$ linkers between pyridinium rings and their reactivity against VX. *Bioorg Med Chem Lett* 16:4852–4855, 2006.
- Oh KA, Park NJ, Park NS, Kuča K, Jun D, Jung YS: Determination of reactivation potency for DFP- and paraoxon-inhibited acetylcholinesterases by pyridinium oximes. *Chem Biol Interact* 175:365–367, 2008.
- Ohta H, Ohmori T, Suzuki S, Ikegaya H, Sakurada K, Takatori T: New safe method for preparation of sarin-exposed human erythrocytes acetylcholinesterase using non-toxic and stable sarin analogue isopropyl p-nitrophenyl methylphosphonate and its application to evaluation of nerve agent antidotes. *Pharm Res* 23:2827–2833, 2006.
- Petroianu GA, Arafat K, Kuča K, Kassa J: Five oximes (K-27, K-33, K-48, BI-6 and methoxime) in comparison with pralidoxime: *in vitro* reactivation of red blood cell acetylcholinesterase inhibited by paraoxon. *J Appl Toxicol* 26:64–71, 2006a.
- Petroianu GA, Nurulain SM, Nagelkerke N, Al-Sultan MAH, Kuča K, Kassa J: Five oximes (K-27, K-33, K-48, BI-6 and methoxime) in comparison with pralidoxime: survival in rats exposed to the organophosphate paraoxon. *J Appl Toxicol* 26:262–268, 2006b.
- Petroianu GA, Hasan MY, Nurulain SM, Nagelkerke N, Kassa J, Kuča K: New K-oximes (K-27 and K-48) in comparison with obidoxime (LuH-6), HI-6, trimedoxime (TMB-4) and pralidoxime (2-PAM): survival in rats exposed ip to the organophosphate paraoxon. *Toxicol Mech Methods* 17:401–408, 2007a.
- Petroianu GA, Nurulain SM, Nagelkerke N, Shafiullah M, Kassa J, Kuča K: Five oximes (K-27, K-48, obidoxime, HI-6 and trimedoxime) in comparison with pralidoxime: survival in rats exposed to methyl-paraoxon. *J Appl Toxicol* 27:453–457, 2007b.
- Petroianu GA, Arafat K, Nurulain SM, Kuča K, Kassa J: *In vitro* oxime reactivation of red blood cell acetylcholinesterase inhibited by methyl-paraoxon. *J Appl Toxicol* 27:168–175, 2007c.
- Pícha J, Kuča K, Kivala M, Kohout M, Cabal J, Liška F: New group of monoquaternary reactivators of the acetylcholinesterase inhibited by nerve agents. *J Enzyme Inhib Med Chem* 20:233–237, 2005.
- Pohanka M, Jun D, Kuča K: Amperometric biosensor for evaluation of competitive cholinesterase inhibition by the reactivator HI-6. *Anal Lett* 40:2351–2359, 2007.
- Singh H, Moorad-Doctor D, Ratcliffe RH, Wachtel K, Castillo A, Garcia GE: A rapid cation-exchange HPLC method for detection and quantification of pyridinium oximes in plasma and tissue. *J Anal Toxicol* 31:69–74, 2007.
- Tekes K, Hasan MY, Sheen R, Kuča K, Petroianu G, Ludanyi K, Kalasz H: HPLC determination of the serum concentration of K-27, a novel oxime-type cholinesterase reactivator. *J Chromatogr A* 1122:84–87, 2006.
- Thiermann H, Szinicz L, Eyer F, Worek F, Eyer P, Felgenhauer N, Zilker T: Modern strategies in therapy of organophosphate poisoning. *Toxicol Lett* 107:233–239, 1999.
- Tu AT: Overview of sarin terrorist attacks in Japan. *ACS Symp Ser Am Chem Soc* 745:304–317, 2000.
- Wiesner J, Kříž Z, Kuča K, Jun D, Koča J: Acetylcholinesterases – the structural similarities and differences. *J Enzyme Inhib Med Chem* 22:417–424, 2007.
- Yang GY, Oh KA, Park NJ, Jung YS: New oxime reactivators connected with $\text{CH}_2\text{O}(\text{CH}_2)_n\text{OCH}_2$ linker and their reactivation potency for organophosphorus agents-inhibited acetylcholinesterase. *Bioorg Med Chem* 15:7704–7710, 2007.
- Žďárová Karasová J, Bajgar J, Novotný L, Kuča K: Is a high dose of Huperzine A really suitable for pretreatment against high doses of soman? *J Appl Biomed* 7:93–99, 2009.

