

ORIGINAL ARTICLE

A comparison of the reactivating and therapeutic efficacy of the newly developed bispyridinium oxime K203 with currently available oximes, in sarin poisoned rats and mice

Jiří Kassa¹, Jana Žďárová Karasová², Vendula Šepsová¹, Jiří Bajgar¹

¹Department of Toxicology, Faculty of Military Health Sciences, University of Defense, Hradec Králové, Czech Republic

²Department of Public Health, Faculty of Military Health Sciences, University of Defense, Hradec Králové, Czech Republic

Received 5th May 2011.

Revised 9th June 2011.

Published online 8th July 2011.

Summary

This study compares the abilities of the newly developed bispyridinium oxime K203 with currently available oximes (HI-6, obidoxime, and trimedoxime) in the reactivation of sarin-inhibited acetylcholinesterase and the reduction of the acute toxicity of sarin. The percentage of reactivation of sarin-inhibited rat blood and tissue acetylcholinesterase was determined *in vivo* and it was shown that the potency of bispyridinium oxime K203 to reactivate sarin-inhibited acetylcholinesterase roughly corresponds to the relatively low reactivating efficacy of obidoxime and trimedoxime except in the diaphragm where K203 was not effective. On the other hand, the oxime HI-6 was found to be a very efficient reactivator of sarin-inhibited acetylcholinesterase in the peripheral as well as central compartment. The oxime HI-6 was able to reduce the acute toxicity of sarin by more than four times, but the other oximes studied, including K203, decreased the acute toxicity of sarin by less than three times. Based on these results, we can conclude that the reactivating and therapeutic efficacy of the oxime K203 is significantly lower compared to the oxime HI-6 and, therefore, it is not a suitable replacement for the oxime HI-6 in the antidotal treatment of acute sarin poisoning.

Key words: sarin; acetylcholinesterase; K203; HI-6; obidoxime; trimedoxime; rats; mice

INTRODUCTION

Highly toxic organophosphorus compounds, called nerve agents, present a serious threat to both military and civilian populations. They irreversibly inhibit the

enzyme acetylcholinesterase (AChE, EC 3.1.1.7) leading to acetylcholine accumulation at synaptic clefts and overstimulation of the muscarinic and nicotinic cholinergic receptors located in the central and peripheral cholinergic nervous system. Severe poisoning can lead to death due to respiratory failure (Marrs 1993, Lotti 2000).

The current antidotal treatment of nerve agent poisoning usually consists of the combined administration of an anticholinergic drug (preferably atropine) and an oxime (preferably pralidoxime or obidoxime). The anticholinergic drug blocks the overstimulation of muscarinic receptor sites, while the oxime repairs biochemical lesions by dephosphorylating nerve agent-inhibited AChE and

✉ Jiří Kassa, Faculty of Military Health Sciences, Třebešská 1575, 500 01 Hradec Králové, Czech Republic

kassa@pmfhk.cz

+420 973 251 500

+420 495 518 094

restoring its activity (Taylor 2001, Kassa 2002). Unfortunately, the oximes are not satisfactorily effective against all nerve agents; in particular, the antidotal treatment of acute poisonings with tabun, cyclosarin and soman is very difficult (Dawson 1994, Kassa 2002, Bajgar 2004) and therefore, the antidotal treatment of nerve agent acute poisonings still remains a serious problem. The development of a new and more effective AChE reactivator is still a very important issue.

The new bispyridinium asymmetric oxime K203 [1-(4-carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide] (Fig. 1) has been synthesized in our department (Musilek et al. 2008). The purpose was to increase the efficacy of antidotal treatment of tabun poisoning which had been shown to be resistant to conventional oxime therapy due to the conformational changes in the AChE-tabun complex prior ageing process at the AChE active site (Ekström et al. 2006). As the oxime K203 has been found to be a promising reactivator of tabun-inhibited AChE (Kassa et al. 2008), we decided to evaluate the reactivating and therapeutic efficacy of K203 against other nerve agents including sarin (isopropyl methylfluorophosphonate), because we are still searching for a broad-spectrum oxime able to sufficiently counteract the acute toxicity of all nerve agents regardless of their chemical structure.

The aim of this study was therefore to determine the reactivating and therapeutic efficacy of the newly developed bispyridinium oxime K203 compared to the currently available oximes (HI-6, obidoxime, trimedoxime) against sarin in rats and mice.

MATERIAL AND METHODS

Animals

Male albino Wistar rats weighing 230–260 g (6 weeks old) and NMRI male mice weighing between 20 and 24 g (4 weeks old) were purchased from Velaz (Prague, Czech Republic). They were housed in polypropylene cages ($56 \times 36 \times 19$ cm³, six rats or ten mice per cage) in an air-conditioned room (22 ± 2 °C and $50 \pm 10\%$ relative humidity, with lights from 7.00 a.m. to 7.00 p.m.) and were allowed access to standard food and tap water *ad libitum*. The laboratory animals were acclimatized in the laboratory vivarium for 14 days before starting the experiments and they were divided into groups of 8 animals. Handling of the experimental animals was performed in compliance with relevant laws and institutional guidelines and with the approval of the

Ethics Committee of the Faculty of Military Health Sciences in Hradec Králové (Czech Republic).

Chemicals

Sarin was obtained from the Military Technical Institute in Brno (Czech Republic) and was 98% pure, as assayed by an acidimetric titration. All oximes (K203, HI-6, trimedoxime, obidoxime) were synthesized at our Department of Toxicology of the Faculty of Military Health Sciences (Czech Republic) and they were more than 98% pure (Musilek et al. 2006, 2008). Their purity was analysed using the HPLC technique (Jun et al. 2007). All other drugs and chemicals of analytical grade were obtained commercially and used without further purification. All substances were administered intramuscularly (i.m.) at a volume of 1 ml/kg body weight (b.w.) to rats and 10 ml/kg b.w. to mice.

In vivo experiments

To investigate the reactivating efficacy of the oximes, the rats were administered i.m. with either atropine (21 mg/kg) alone or atropine (21 mg/kg) in combination with one of the studied oximes at 5 min before receiving sarin i.m. at a dose of 90 µg/kg (LD_{50}). The oxime K203 (16.3 mg/kg), the oxime HI-6 (39.3 mg/kg), obidoxime (10.5 mg/kg) and trimedoxime (7.5 mg/kg) were administered in equitoxic doses corresponding to 5% of their previously published LD_{50} values (Kassa et al. 2008). The rats were decapitated and exsanguinated to obtain the blood 30 min after sarin poisoning. The blood was haemolysed in Tris-HCl buffer (0.02 N; pH 7.6; 1:20). The tissues, diaphragm and brain were removed and homogenized in Tris-HCl buffer (0.02 N; pH 7.6; 1:10) to determine AChE activity by the standard spectrophotometric method (Ellman et al. 1961). Acetylthiocholine was used as a substrate (0.1 N Tris-HCl buffer; pH 7.6). A Helios Alpha spectrophotometer (Thermo Electron Corporation, UK), was used for the determination of absorbancy at 436 nm. The AChE activity was derived from absorbance values with the help of the calibration curve with cysteine and expressed as µkat/kg or l (µmol substrate hydrolyzed/kg wet tissue or l blood within 1 second). The percentage of reactivation was calculated using the AChE activity values: $\{1 - [(saline\ control) - (oxime + atropine)] / [(saline\ control) - (atropine\ control)]\} \times 100$ (equation modified from Clement et al. 1992).

The ability of oximes in combination with atropine to decrease the acute toxicity of sarin in mice was determined as follows. The LD_{50} value of sarin and its 95% confidence limit in mice were assessed

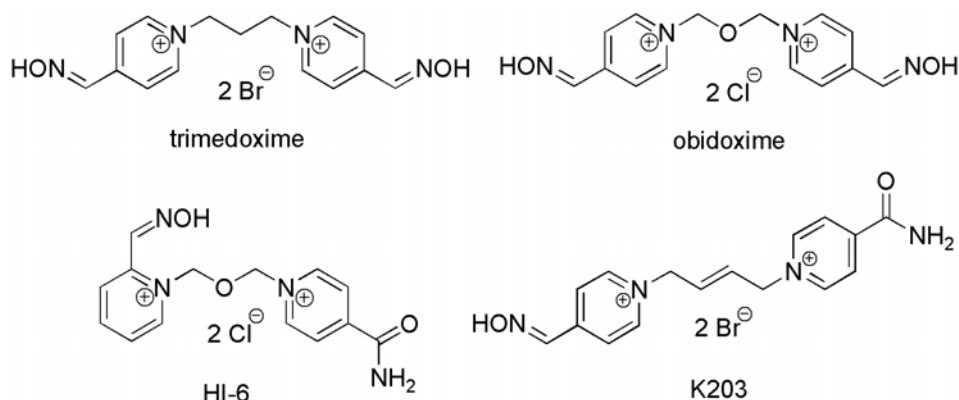


Fig. 1. Chemical structure of oximes.

using probit-logarithmical analysis of death occurring within 24 h after i.m. administration of sarin at five different doses with eight mice per dose (Tallarida and Murray 1987). Sarin-poisoned mice were then treated i.m. with one of the tested oximes in combination with atropine (21 mg/kg) 1 min after i. m. challenge of sarin. The oxime K203 (4.75 mg/kg), the oxime HI-6 (33.6 mg/kg), obidoxime (9.4 mg/kg) and trimedoxime (7.4 mg/kg) were administered in equitoxic doses corresponding to 5% of their previously published LD_{50} values (Kassa et al. 2008). The efficacy of the tested antidotal treatment was expressed as a protective ratio (LD_{50} value of sarin in protected mice/ LD_{50} value of sarin in unprotected mice).

Statistical evaluation

The differences between groups were calculated using means \pm SD and differences were tested by one-way ANOVA test with Scheffe's post hoc test at the significance level $2\alpha=0.05$.

RESULTS

The potency of oximes to reactivate sarin-inhibited AChE in rat blood, diaphragm and brain *in vivo* is shown in Table 1. The oxime K203 is a relatively weak reactivator of sarin-inhibited AChE. It was able to increase the activity of sarin-inhibited AChE in blood by 6.3% and in brain by 3.1% while its ability to reactivate sarin-inhibited AChE in the diaphragm was negligible. Its potency to reactivate sarin-inhibited AChE was comparable to the reactivating efficacy of obidoxime and trimedoxime with the exception of the diaphragm where the

potency of obidoxime and trimedoxime to reactivate sarin-inhibited AChE was significantly higher. All the above mentioned oximes are markedly weaker reactivators of sarin-inhibited AChE than the oxime HI-6 which seems to be the most effective reactivator of sarin-inhibited AChE in the peripheral as well as central compartment; it was able to increase the activity of sarin-inhibited AChE by more than 70% in blood, by almost 50% in the diaphragm and by 19% in the brain. The differences between HI-6 and the other oximes studied (K203, obidoxime, and trimedoxime) in the blood, the diaphragm as well as the brain were significant (Table 1).

The potency of oximes studied in reducing the acute toxicity of sarin is shown in Table 2. The oxime K203 was able to decrease the acute toxicity of sarin by almost 2.5 times. Its therapeutic efficacy roughly corresponds to the potency of obidoxime and trimedoxime in decreasing the acute toxicity of sarin. On the other hand, the oxime HI-6 showed significantly higher potency in the reduction of the acute lethal toxic effects of sarin in mice than all other oximes studied. It decreased the acute toxicity of sarin by more than four times (Table 2).

DISCUSSION

Due to the threat of misuse of various nerve agents for military as well as terrorist purposes, a broad-spectrum oxime, sufficiently effective against nerve agents regardless of their chemical structure, is urgently required as a satisfactorily effective antidotal treatment of nerve agent exposure. Generally, it is known that no currently available oxime is able to satisfactorily reactivate the AChE inhibited by all

Table 1. Percentage of reactivation of sarin-inhibited AChE by oximes in rat blood, diaphragm and brain *in vivo*.

Treatment	AChE activity (μkat/l or μkat/kg)		
	Blood	Diaphragm	Brain
Atropine	6.83±0.62 ^a	5.69±1.42 ^a	27.29±3.51 ^a
Atropine + K203 (% reactivation ^b)	7.43±0.49 (6.3)	5.37±1.12 (0)	31.39±3.69 (3.1)
Atropine + HI-6 (% reactivation)	13.69±1.41 (71.6* ^x)	10.88±1.39 (48.7* ^x)	52.49±4.38 (19.3* ^x)
Atropine + obidoxime (% reactivation ^b)	7.35±0.96 (5.4)	6.16±0.95 (4.4)	33.42±9.29 (4.6)
Atropine + trimedoxime (% reactivation)	7.66±0.54 (8.6)	6.92±1.16 (11.6)	36.41±7.19 (6.9)

^a means ± SD, N = 8. The untreated control value (saline control) for rat blood AChE activity was 16.41 μkat/l, for diaphragm AChE activity 16.35 μkat/kg and for brain AChE activity 157.60 μkat/kg

^b percent reactivation was determined using the AChE activity values: $\{1 - [((\text{saline control}) - (\text{oxime} + \text{atropine})) / ((\text{saline control}) - (\text{atropine control}))]\} \times 100$

* significantly different from the atropine group

^x significantly different from the atropine + obidoxime (trimedoxime, K203) group

Table 2. The influence of the type of oxime on the potency of antidotal treatment to reduce acute toxicity of sarin in mice.

Treatment	LD ₅₀ (μg/kg)±95% confidence limit	Protective ratio
–	116.1 (96.8–139.3)	–
K203 + atropine	271.0 (217.3–311.2)*	2.33
HI-6 + atropine	523.3 (423.0–617.1)* ^x	4.56
Obidoxime + atropine	334.0 (272.6–378.5)*	2.88
Trimedoxime + atropine	308.4 (265.6–338.1)*	2.66

* significantly different from the untreated group

^x significantly different from the group treated by atropine in combination with obidoxime (trimedoxime, K203)

nerve agents (Marrs et al. 2006, Kassa et al. 2007, Szinicz et al. 2007). The oxime HI-6 seems to be the most effective and broadest oxime among the commonly used oximes, but it is a weak reactivator of tabun-inhibited AChE (Lundy et al. 2006, Kuča et al. 2009). Therefore, new structural analogues of currently available oximes have been developed to find a broad-spectrum reactivator of nerve agent-inhibited AChE (Kuca et al. 2004, 2006). Their reactivating efficacy depends on the chemical structure of a linker connecting both pyridinium rings (in the case of bispyridinium oximes), the position of

an oxime group, the chemical structure and the position of a functional group situated on the non-oximated pyridinium ring (Kuca et al. 2006, 2007, Jokanović and Prostran 2009). On the other hand, a higher number of aldoxime groups is not necessary. The oxime HI-6 has only one oxime group and it is significantly more efficacious to reactivate soman and cyclosarin-inhibited AChE than bispyridinium oximes with two oxime groups such as obidoxime, methoxime or trimedoxime (Kassa and Cabal 1999a, b, Lundy et al. 2006).

The oxime K203 was primarily synthesized to increase the effectiveness of the antidotal treatment of tabun poisonings. *In vivo* evaluation of its reactivating efficacy against tabun showed that K203 is a really promising oxime for the antidotal treatment of acute tabun poisonings (Kassa et al. 2008, Kovarik et al. 2009). Unfortunately, based on the results obtained, its potency to reactivate sarin-inhibited AChE is relatively low and similar to the reactivating efficacy of obidoxime and trimedoxime, probably due to its chemical structure that is advantageous for its potency in reactivating tabun-inhibited AChE, but is disadvantageous for their potency in reactivating AChE inhibited by fluorophosphonates such as soman, sarin and cyclosarin (Kuca et al. 2006, 2007). While the potency of bispyridinium oximes with the oxime groups in position 4 (obidoxime, trimedoxime, K203) in reactivating sarin-inhibited AChE is relatively low, the oxime HI-6 with the oxime group in position 2 showed the high potency in reactivating sarin-inhibited AChE in the peripheral as well as the central compartment and in reducing its acute toxicity.

The findings clearly showed that the oxime K203 as well as all other currently available oximes are not suitable as broad-spectrum reactivators of nerve agent-inhibited AChE. Therefore, the combination of two oximes with a different spectrum of their reactivating and therapeutic efficacy seems to be a possible approach to increasing the efficacy of antidotal treatment of acute poisonings with nerve agents regardless of their chemical structure (Clement et al. 1987, Maksimović and Kovačević 1989, Kovačević et al. 1991, Worek et al. 2007). To combine two oximes with a different spectrum of high reactivating efficacy seems to be an appropriate way to cover the whole spectrum of nerve agents.

Although the potency of K203, obidoxime and trimedoxime in reactivating sarin-inhibited AChE in rats is relatively low, they are able to reduce the acute toxicity of sarin in mice by more than twice, probably due to other beneficial effects than reactivation of sarin-inhibited AChE. It has previously been demonstrated that oximes are able to directly influence the activity of muscarinic as well as nicotinic receptors (Van Helden et al. 1996, Soukup et al. 2010).

The results obtained confirm that none of the oximes studied can be considered as a broad-spectrum reactivator able to sufficiently counteract acute toxicity of all nerve agents regardless of their chemical structure. The newly developed bispyridinium oxime K203, satisfactorily effective against tabun (Kassa et al. 2008), was not found to be sufficiently effective against sarin. In contrast, the

oxime HI-6 is a much more effective oxime for the reactivation of sarin-inhibited AChE in rats and for the reduction of acute toxicity of sarin in mice than all the other oximes studied. Therefore, it is still the most promising oxime for the antidotal treatment of acute sarin poisoning.

DECLARATION OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

ACKNOWLEDGEMENTS

The authors would like to thank Mrs. J. Uhlířová for her skilful technical assistance. The study was supported by the grant of Ministry of Defence, MO0FVZ0000501.

REFERENCES

- Bajgar J. Organophosphate/nerve agent poisoning: mechanism of action, diagnosis, prophylaxis, and treatment. *Adv Clin Chem.* 38: 151–216, 2004.
- Clement JG, Shiloff JD, Gennings Ch. Efficacy of a combination of acetylcholinesterase reactivators, HI-6 and obidoxime, against tabun and soman poisoning of mice. *Arch Toxicol.* 61: 70–75, 1987.
- Clement JG, Hansen AS, Boulet CA. Efficacy of HLö-7 and pyrimidoxime as antidotes of nerve agent poisoning in mice. *Arch Toxicol.* 66: 216–219, 1992.
- Dawson RM. Review of oximes available for treatment of nerve agent poisoning. *J Appl Toxicol.* 14: 317–331, 1994.
- Ekström F, Akfur C, Tunemalm AK, Lundberg S. Structural changes of phenylalanine 338 and histidine 447 revealed by the crystal structures of tabun-inhibited murine acetylcholinesterase. *Biochemistry.* 45: 74–81, 2006.
- Ellman GL, Courtney DK, Andres V, Jr., Featherstone RM. A new and rapid determination of acetylcholinesterase activity. *Biochem Pharmacol.* 7: 88–93, 1961.
- Jokanović M, Prostran M. Pyridinium oximes as cholinesterase reactivators. Structure-activity relationship and efficacy in the treatment of

- poisoning with organophosphorus compounds. *Curr Med Chem*. 16: 2177–2188, 2009.
- Jun D, Kuca K, Stodulka P, Koleckar V, Dolezal B, Simon P, Veverka M. HPLC analysis of HI-6 dichloride and dimethanesulfonate – antidotes against nerve agents and organophosphorus pesticides. *Anal Lett*. 40: 2783–2787, 2007.
- Kassa J. Review of oximes in the antidotal treatment of poisoning by organophosphorus nerve agents. *J Toxicol Clin Toxicol*. 40: 803–816, 2002.
- Kassa J, Cabal J. A comparison of the efficacy of a new asymmetric bispyridinium oxime BI-6 with currently available oximes and H oximes against soman by *in vitro* and *in vivo* methods. *Toxicology*. 132: 111–118, 1999a.
- Kassa J, Cabal J. A comparison of the efficacy of acetylcholinesterase reactivators against cyclohexyl methylphosphonofluoridate (GF agent) by *in vitro* and *in vivo* methods. *Pharmacol Toxicol*. 84: 41–45, 1999b.
- 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. *Cur Org Chem*. 11: 267–283, 2007.
- Kassa J, Karasová J, Musílek K, Kuča K. An evaluation of therapeutic and reactivating effects of newly developed oximes (K156, K203) and commonly used oximes (obidoxime, trimedoxime, HI-6) in tabun-poisoned rats and mice. *Toxicology*. 243: 311–316, 2008.
- Kovacević V, Maksimović M, Deljać V, Binenfeld Z. Protective effects of mixture of oximes in poisoning by nerve chemical warfare agents. *Acta Pharm Jugosl*. 41: 75–78, 1991.
- Kovarik Z, Vrdoljak AL, Berend S, Katalinić M, Kuča K, Musílek K, Radić B. Evaluation of oxime K203 as antidote in tabun poisoning (In Croatian). *Arh Hig Rada Toksikol*. 60: 19–26, 2009.
- Kuča K, Cabal J, Kassa J. A comparison of the efficacy of a bispyridinium oxime – 1,4-bis-(2-hydroxyiminomethylpyridinium) butane dibromide and currently used oximes to reactivate sarin, tabun or cyclosarin-inhibited acetylcholinesterase by *in vitro* methods. *Pharmazie*. 59: 795–798, 2004.
- Kuca K, Jun D, Musílek K. Structural requirements of acetylcholinesterase reactivators. *Mini Rev Med Chem*. 6: 269–277, 2006.
- Kuca K, Racakova V, Jun D, Bajgar J. Structure-activity relationship of acetylcholinesterase reactivators – antidotes against nerve agents. *Lett Org Chem*. 4: 212–217, 2007.
- Kuča K, Musílek K, Jun D, Pohanka M, Žďárová Karasová J, Novotný L, Musilová L. Could oxime HI-6 really be considered as “broad-spectrum” antidote? *J Appl Biomed*. 7: 143–149, 2009.
- Lotti M. Organophosphorus compounds. In Spencer PS, Schaumburg HH (eds.): *Experimental and Clinical Neurotoxicology*, Oxford University Press, New York 2000, pp. 898–925.
- Lundy PM, Raveh L, Amitai G. Development of the bisquaternary oxime HI-6 toward clinical use in the treatment of organophosphate nerve agent poisoning. *Toxicol Rev*. 25: 231–243, 2006.
- Maksimović M, Kovačević V. Protective and reactivating effects of HI-6-toxogonin mixture in rats and guinea-pigs poisoned by nerve agents. *Acta Pharm Jugosl*. 39: 27–33, 1989.
- Marrs TC. Organophosphate poisoning. *Pharmacol Ther*. 58: 51–66, 1993.
- Marrs TC, Rice P, Vale JA. The role of oximes in the treatment of nerve agent poisoning in civilian casualties. *Toxicol Rev*. 25: 297–323, 2006.
- Musílek K, Kuča K, Jun D, Doležal M. Synthesis of bispyridinium compounds bearing propane linker and evaluation of their reactivation activity against tabun- and paraoxon-inhibited acetylcholinesterase. *Lett Org Chem*. 3: 831–835, 2006.
- Musílek K, Holas O, Kuča K, Jun D, Dohnal V, Opletalová V, Doležal M. Synthesis of monooxime-monocarbamoyl bispyridinium compounds bearing (E)-but-2-ene linker and evaluation of their reactivation activity against tabun- and paraoxon-inhibited acetylcholinesterase. *J Enzyme Inhib Med Chem*. 23: 70–76, 2008.
- Soukup O, Holas O, Binder J, Killy K, Tobin G, Jun D, Fusek J, Kuča K. The effect of trimedoxime on acetylcholinesterase and on the cholinergic system of the rat bladder. *J Appl Biomed*. 8: 87–92, 2010.
- Szinicz L, Worek F, Thiermann H, Kehe K, Eckert S, Eyer P. Development of antidotes: problems and strategies. *Toxicology*. 233: 23–30, 2007.
- Tallarida R, Murray R. *Manual of Pharmacological Calculation with Computer Programs*. Springer-Verlag, New York 1987.
- Taylor P. Anticholinesterase agents. In Hardman JG, Limbird LE (eds.): *The Pharmacological Basis of Therapeutics*, McGraw Hill, New York 2001, pp. 175–191.
- Van Helden HPM, Busker RW, Melchers BPC, Bruijnzeel PLB. Pharmacological effects of oximes: how relevant are they? *Arch Toxicol*. 70: 779–786, 1996.
- Worek F, Aurbek N, Thiermann H. Reactivation of organophosphate-inhibited human AChE by combinations of obidoxime and HI 6 *in vitro*. *J Appl Toxicol*. 27: 582–588, 2007.