Strategies for the Development of Effective Broad-Spectrum Oximes

Dr. Franz Worek, COL(MC) and Dr. Horst Thiermann, COL(MC)
Bundeswehr Institute of Pharmacology and Toxicology
Neuherbergstrasse 11, 80937 Munich, Germany
Phone +49-89-3168-2930, Fax +49-89-3168-2333

franzworek@bundeswehr.org

ABSTRACT

Highly toxic organophosphorus-type (OP) chemical warfare agents ("nerve agents") and OP pesticides may be used by terrorists and during military conflicts emphasizing the necessity for the development of effective medical countermeasures. Presently, standard treatment consists of atropine and acetylcholinesterase (AChE) reactivators ("oximes"). Hereby, the widely used oximes obidoxime and pralidoxime (2-PAM) are considered to be ineffective with certain nerve agents. In the past decades numerous oximes have been synthesized with a focus on higher efficacy against soman poisoning. However, the anticipated scenarios of asymmetric warfare and the increasing likelihood of terrorist use of OP, either nerve agents, nerve agent derivatives or pesticides, calls for the development of broad spectrum oximes covering the whole range of potential threat agents. In order to meet this goal different strategies are conceivable: Development of newly synthesized oximes with a sufficient efficacy against the range of threat agents or a combined use of two or more oximes having a complementary spectrum. An overview is given on recent studies on the reactivation of OP-inhibited human AChE by oximes. Almost fifty structurally different oximes were tested on their ability to reactivate human AChE inhibited by GA, GF or VX. The results of this study indicate that various bispyridinium oximes were effective reactivators at high concentration but only few compounds were effective against all tested nerve agents. The ability of obidoxime and HI 6 combinations to reactivate human AChE inhibited by GA, GB, GF, VX and paraoxon was tested at human relevant oxime concentrations. The rationale for using this oxime combination was the fact that HI 6 is an effective reactivator of GB-, GF- and VX-inhibited AChE while obidoxime is effective in case of paraoxon and a reasonable reactivator of GA-inhibited AChE. Two major findings of this study were that a combination of HI 6 and obidoxime did not impair reactivation, compared to HI 6 or obidoxime alone, but broadened the spectrum compared to the individual oximes. At present, none of the oximes tested so far can be considered as real broad spectrum reactivator covering the whole range of threat agents. Hence, further research is necessary to fill this gap. A short-term alternative could be the combined use of two or more oximes having a complementary spectrum.

1.0 INTRODUCTION

Despite of long-lasting efforts by the international community, leading to the Chemical Weapons Convention that came into force in 1997, highly toxic organophosphorus warfare agents ("nerve agents") are stockpiled by different countries and pose a potential threat to military forces. Several incidents of terrorist use of the nerve agents sarin and VX against civilians in Matsumoto and Tokyo in 1994 and 1995 (Moriata et al. 1995; Nagao et al. 1997; Nozaki et al. 1995) underline the necessity for the development and availability of effective medical countermeasures against such agents.

The mechanism of action of organophosphorus compounds (OP), i.e. nerve agents and pesticides, is attributed to the covalent binding of these compounds to the active site of the enzyme acetylcholinesterase

Worek, F.; Thiermann, H. (2007) Strategies for the Development of Effective Broad-Spectrum Oximes. In *Defence against the Effects of Chemical Hazards: Toxicology, Diagnosis and Medical Countermeasures* (pp. 30-1 – 30-6). Meeting Proceedings RTO-MP-HFM-149, Paper 30. Neuilly-sur-Seine, France: RTO. Available from: http://www.rto.nato.int.

(AChE; EC 3.1.1.7) leading to a disturbance of its physiologic function, the hydrolyzing of the neurotransmitter acetylcholine at central and peripheral synapses (Holmstedt 1959; Taylor et al. 1995). Acetylcholine accumulation cuases a pathologic over-stimulation of cholinergic receptors which impacts numerous body functions and finally results in respiratory arrest and death.

Presently, standard treatment of OP poisoning includes the administration of a muscarinic antagonist, e.g. atropine, and of an oxime to reactivate inhibited AChE. Hereby, anti-muscarinic drugs act only symptomatically while oximes may restore the enzyme function by removing the phosphyl (denotes phosphonyl and phosphoryl) moiety from the active site. Hence, these compounds can be expected to act as specific antidotes (Hobbiger 1963; Eyer and Worek 2007). Unfortunately, the established oximes obidoxime, pralidoxime and TMB-4 are considered to be rather ineffective against various nerve agents. Numerous new oximes were synthesized in the past decades (Reiner et al. 2006). In former times the search for more effective oximes was primarily directed towards soman poisoning. The increasing risk of terrorist use of OP (nerve agents and pesticides) resulted in a shift of research efforts towards compounds that may serve as broad-spectrum reactivators. Presently, several bispyridinium oximes (e.g. HI 6, HLö 7, MMB-4) are under investigation to replace the established compounds. In addition, numerous other oxime-based reactivators, in part bearing imidazolium and quinuclidinium groups, have been synthesized in laboratories in the USA (Bedford et al. 1986; Kenley et al. 1985; Bedford et al 1984; Kenley et al. 1984; Kenley et al. 1981), Israel (Amitai et al. 1995), Croatia (Reiner et al. 2006; Primozic et al. 2004) and in the Czech Republic (Musilek et al. 2006). This list is by no means complete but comprises probably the compounds of broader interest as published in the open literature.

The potential replacement of the established oximes obidoxime and pralidoxime by HI 6 or MMB-4 will not result in the availability of an antidote covering the whole spectrum of threat agents (Worek et al. 2007a). The major drawback of HI 6 is its weak reactivating potency with pesticide-inhibited AChE and its failure to reactivate tabun-inhibited AChE (Worek et al. 2004). MMB-4 was shown to be superior to pralidoxime but mostly less potent than obidoxime and HI 6. Hence, the next generation of oxime antidotes (HI 6 or MMB-4) will give some improvement but will not fulfill the requirement as broad-spectrum reactivators. This calls for the search for new, more effective reactivators and potential approaches for meeting this goal will be discussed.

2.0 DEVELOPMENT OF OXIMES: THE KINETIC APPROACH

Numerous studies investigated the mechanism of oxime action in the past decades (Hobbiger 1963; Eyer 1996; Hagedorn et al. 1969; Ashani et al. 1971, Su et al. 1983; Wong et al. 2000; Kovarik et al. 2004; Eyer and Worek 2007) and there is convincing evidence that the main mechanism of action of oximes is reactivation of inhibited AChE, i.e. removal of the phosphyl moiety from the active site serine. Reactivation proceeds via a two-step reaction, first forming a fully reversible Michaelis-type phosphyl-AChE-complex followed by the removal of the phosphyl residue. The efficacy of the reactivator is attributed to the nucleophilicity of the oximate and the decay rate of the intermediate phosphyloxime and is dependent on the structure of the oxime and of the OP moiety as well as on the architecture of the enzyme. Hence, in vitro studies investigating the reactivation kinetics of oximes may contribute substantially to the assessment of oxime efficacy. Hereby, human AChE should be used since previous investigations showed marked differences between human and animal AChE (Worek et al. 2002).

In order to get insight into the structural requirements of oximes to reactivate OP-inhibited AChE it is indispensable to determine the reactivation rate constants for a variety of oximes and inhibitors. Data from previous studies indicated that an oxime function at position 2 is important for the reactivation of soman inhibited AChE while 4-oximes seemed to be superior to 2-oximes with OP pesticides and phosphoramidates (De Jong et al. 1989). These findings concerning OP pesticides and phosphoramidates

were confirmed in a recent studies, using a large number of different oximes. However, that the effect of position of the oxime group on the reactivation of phosphonylated AChE was inconsistent (Worek et al. 2004 and unpublished data). The 2-oxime HI 6 showed to be an effective reactivator of phosphonylated AChE, on the other hand obidoxime, bearing an oxime function at position 4 of both pyridinium rings, was also an excellent reactivator of phosphonylated AChE, except of cyclosarin-inhibited enzyme. Further studies with an extended series of methylfluorophosphonates bearing n- and i-alkyl groups showed again that obidoxime was in part more potent than HI 6 (Bartling et al. 2007). On the other hand no structure-activity relationship between the structure of the alkyl moiety and the reactivating potency of obidoxime, pralidoxime, HI 6, HLö 7 and MMB-4 could be figured out. In contrast, a clear structure-activity of oxime reactivation kinetics could be determined with AChE inhibited by N-monoalkyl tabun analogues depending on the chain length of the N-alkyl residue (Worek et al. 2007b). Interestingly, HI 6 was able to reactivate human AChE inhibited by these phosphoramidates.

The present data on reactivation kinetics of OP-inhibited human AChE do not provide a clear basis for predicting structural requirements of a new broad-spectrum reactivator. Hence, it will be necessary to test candidate oximes with the whole range of threat agents in order to select a promising new reactivator.

3.0 DEVELOPMENT OF OXIMES: THE STRUCTURAL APPROACH

The resolution of the three-dimensional structure of AChE from Torpedo californica (Sussmann et al. 1991) was the starting point for detailed structural studies. By using site-directed mutagenesis and molecular modeling, a number of studies were undertaken in order to unravel the underlying structural properties determining the mechanisms for inhibition and reactivation of the enzyme (Taylor et al. 1999). Experiments with recombinant mouse AChE and mutant enzymes using different inhibitors and oximes gave further insight into the structural components involved in the interactions between the active center gorge, the inhibitor and the oxime (Wong et al. 2000; Luo et al. 2003; Kovarik et al. 2004; Ekström et al. 2006a+b). Such studies could provide important data on the structural requirements of effective reactivators and could thus reduce the number of necessary kinetic experiments.

However, one has to keep in mind that at present only crystal structures of non-human AChE are available. Although it is assumed that the primary structure of mammalian AChE is very similar kinetic data of inhibition, reactivation and aging from AChE of various species, including man, showed marked differences. Hence, it is indispensable to resolve the crystal structure of native and OP-inhibited human AChE in order to elucidate the structural requirements of a promising reactivator.

4.0 DEVELOPMENT OF OXIMES: THE PRAGMATIC APPROACH

In case of selection of promising reactivators by structural analysis and kinetic studies numerous further investigations are necessary for the development of a reactivator to a licensed drug. Further pharmacological and toxicological testing, in vivo efficacy experiments in different animal species and phase I clinical studies in human volunteers will be required in order to get approval by the authorities. Hence, it will take several years between identification of a prospective reactivator and availability as an antidote for human use.

This time line requires the consideration of interim solutions. A conceivable approach could be a combination of oximes which are already marketed or at an advanced stage of development. Hereby, two or more oximes with a complementary activity spectrum could be selected. Unfortunately, only limited data are available in the open literature. In vivo studies with different species showed that a combination of obidoxime and HI 6 resulted in an increased protective ratio compared to single oxime administration in

sarin, tabun and VX poisoning (Clement et al., 1987; Maksimovic and Kovacevic 1989). A recent in vitro study with sarin-, cyclosarin-, tabun- VX- and paraoxon-inhibited human AChE demonstrated that a combination of human relevant concentrations of obidoxime and HI 6 broadened the spectrum compared to obidoxime and HI 6 alone (Worek et al. 2007c). In addition, the results of this study indicated that in a combined use of obidoxime and HI 6 it could be possible to reduce the HI 6 dose which could have beneficial effects with wet-dry autoinjectors in respect of dissolution of the oximes and tolerance at the injection site.

Compared to the development of totally new reactivators obidoxime/HI 6 combinations could be available at a substantially shorter period of time. With such a combination a wide range of potential threat agents would be covered.

5.0 CONCLUSION

At present, none of the oximes tested so far can be considered as real broad spectrum reactivator covering the whole range of threat agents. Hence, further research is necessary to fill this gap. Extensive research using structural analysis, AChE mutants and extensive kinetic studies, will be necessary to identify promising AChE reactivators. A short-term alternative could be the combined use of two or more oximes having a complementary spectrum. A combination of obidoxime and HI 6 is considered to be a promising approach.

[1] Amitai, G., Rabinovitz, I., Zomber, G., Chen, R., Cohen, G., Adani, R., Raveh, L., 1995. Antidotal efficacy of the bisquaternary oximes: AB-8, AB-13, toxogonin, HI-6 and HLö-7 against tabun and soman poisoning. Proceedings 5th International Symposium Protection Against Chemical and Biological Warfare Agents, Stockholm, Sweden, 11-16 June 1995 247-254.

[2] Ashani, Y., and Cohen, S., 1971. Nucleophilicity of some reactivators of phosphorylated acetylcholinesterase. J. Med. Chem. 14, 621-626.

[3] Bartling, A., Worek, F., Szinicz, L., Thiermann, H., 2007. Enzyme-kinetic investigation of different sarin analogues reacting with human acetylcholinesterase and butyrylcholinesterase. Toxicology 233, 166-172.

[4] Bedford, C. D., Harris, R. N., Howd, R. A., Miller, A., Nolen, H. W., Kenley, R. A., 1984. Structure-activity relationships for reactivators of organophosphorus-inhibited acetylcholinesterase: quaternary salts of 2-[(hydroxyimino)methyl]imidazole. J. Med. Chem. 27, 1431-1438.

[5] Bedford, C. D., Howd, R. A., Dailey, O. D., Miller, A., Nolen, H. W., Kenley, R. A., Kern, J. R., Winterle, J. S., 1986. Nonquaternary cholinesterase reactivators. 3. 3(5)-substituted 1,2,4-oxadiazol-5(3)-aldoximes and 1,2,4-oxadiazole-5(3)-thiocarbohydroximates as reactivators of organophosphonate-inhibited eel and human acetylcholinesterase in vitro. J. Med. Chem. 29, 2174-2183.

[6] Clement, J.G., Shiloff, J.D., Gennings, C., 1987. Efficacy of a combination of acetylcholinesterase reactivators, HI-6 and obidoxime, against tabun and soman poisoning of mice. Arch. Toxicol. 61, 70-75.

[7] de Jong, L.P.A., Verhagen, A.A.V., Langenberg, J.P., Hagedorn, I., Löffler, M., 1989. The bispyridinium-dioxime HLö-7: A potent reactivator for acetylcholinesterase inhibited by the stereoisomers of tabun and soman. Biochem. Pharmacol. 38, 633-40.

[8] Ekström, F., Pang, Y.P., Boman, M., Artursson, E., Akfur, C., Börjegren, S., 2006a. Crystal structures of acetylcholinesterase in complex with HI-6, Ortho-7 and obidoxime: Structural basis for differences in the ability to reactivate tabun conjugates. Biochem. Pharmacol. 72, 597-607.

[9] Ekström, F., Akfur, C., Tunemalm, A.K., Lundberg, S., 2006b. Structural changes of phenylalanine 338 and histidine 447 revealed by the crystal structures of tabun-inhibited murine acetylcholinesterase. Biochemistry 45, 74-81.

[10] Eyer, P. (1996). Optimal oxime dosage regimen, a pharmacokinetic approach. In: Role of oximes in the treatment of anticholinesterase agent poisoning (L. Szinicz, P. Eyer, and R. Klimmek, Eds.), pp. 33-51. Spektrum Akademischer Verlag, Heidelberg.

[11] Eyer, P., Worek, F. (2007). Oximes. In: Chemical warfare agents (T.C. Marrs, R.L. Maynard, F. R. Sidell, Eds.), pp. 305-329. John Wiley & Sons, Chichester.

[12] Hagedorn, I., Gündel, W. H., Schoene, K., 1969. Reaktivierung phosphorylierter Acetylcholin-Esterase mit Oximen: Beitrag zum Studium des Reaktionsablaufes. Drug Res. 19, 603-606.

[13] Hobbiger, F. (1963). Reactivation of phosphorylated acetylcholinesterase. In Cholinesterases and anticholinesterase agents (G. B. Koelle, Ed.), pp. 921-988. Springer-Verlag, Berlin.

[14] Holmstedt, B., 1959. Pharmacology of organophosphorus cholinesterase inhibitors. Pharmacol. Rev. 11, 567-688.

[15] Kenley, R. A., Bedford, C. D., Dailey, O. D., Howd, R. A., Miller, A., 1984. Nonquaternary cholinesterase reactivators. 2. □-heteroaromatic aldoximes and thiohydroximates as reactivators of ethyl methylphosphonyl-acetylcholinesterase in vitro. J. Med. Chem. 27, 1201-1211.

[16] Kenley, R. A., Bedford, C. D., Howd, R. A., Jackson, S. E., 1985. Reactivation of ethyl methylphosphonylated eel acetylcholinesterase in vitro by 2-PAM, HI-6 and a series of nonquaternary alpha-ketothiohydroximates. Biochem. Pharmacol. 34, 3606-3608.

[17] Kenley, R. A., Howd, R. A., Mosher, C. W., Winterle, J. S., 1981. Nonquaternary cholinesterase reactivators. Dialkylaminoalkyl thioesters of □-ketothiohydroximic acids as reactivators of diisopropyl phosphorofluoridate inhibited acetylcholinesterase. J. Med. Chem. 24, 1124-1133.

[18] Kovarik, Z., Radic, Z., Berman, H.A., Simeon-Rudolf, V., Reiner, E., Taylor, P., 2004. Mutant cholinesterases possessing enhanced capacity for reactivation of their phosphonylated conjugates. Biochemistry 43, 3222-9.

[19] Luo, C., Leader, H., Radic, Z., Maxwell, D. M., Taylor, P., Doctor, B. P., Saxena, A., 2003. Two possible orientations of the HI-6 molecule in the reactivation of organophosphate-inhibited acetylcholinesterase. Biochem. Pharmacol. 66, 387-392.

[20] Maksimovic, M., Kovacevic, V., 1989. Protective and reactivating effects of HI-6-Toxogonin mixture in rats and guinea-pigs poisoned by nerve agents. Acta Pharmacol. Jugoslav. 39, 27-33.

[21] Morita, H., Yanagisawa, N., Nakajima, T., Shimizu, M., Hirabayashi, H., Okudera, H., Nohara, M., Midorikawa, Y., Mimura, S., 1995. Sarin poisoning in Matsumoto, Japan. Lancet 346, 290-293.

[22] Musilek, K., Kuca, K., Jun, D., Dohnal, V., Dolezal, M., 2006. Synthesis of the novel series of bispyridinium compounds bearing (*E*)-but-2-ene linker and evaluation of their reactivation activity against chlorpyrifos-inhibited acetylcholinesterase. Bioorg. Med. Chem. Lett. 16, 622-627.

[23] Nagao M, Takatori T, Matsuda Y, Nakajima M, Iwase H, Iwadate K. 1997. Definitive evidence for the acute sarin poisoning diagnosis in the Tokyo subway. Toxicol. Appl. Pharmacol. 144, 198-203.

[24] Nozaki, H., Aikawa, N., Fujishima, S., Suzuki, M., Shinozawa, Y., Hori, S., Nogawa, S., 1995. A case of VX poisoning and the difference from sarin. Lancet 346, 698-699.

[25] Primozic, I., Odzak, R., Tomic, S., Simeon-Rudolf, V., Reiner, E., 2004. Pyridinium, imidazolium, and quinuclidinium oximes: Synthesis, interaction with native and phosphylated cholinesterases, and antidotes against organophosphorus compounds. J. Med. Chem. Def. 2, 1-30.

[26] Reiner, E., Simeon-Rudolf, V., 2006. Pyridinium, imidazolium and quinuclidinium compounds: Toxicity and antidotal effects against the nerve agents tabun and soman. Arh. Hig. Rada. Toksikol. 57, 171-179.

[27] Su, C. T., Tang, C. P., Ma, C., Shih, Y. S., Liu, C. Y., Wu, M. T., 1983. Quantitative structure-activity relationships and possible mechanisms of action of bispyridinium oximes as antidotes against pinacolyl methylphosphonofluoridate. Fundam. Appl. Toxicol. 3, 271-277.

[28] Taylor, P., Radic, Z., Hosca, N. A., Camp, S., Marchot, P., Berman, H. A., 1995. Structural basis for the specificity of cholinesterase catalysis and inhibition. Toxicol. Lett. 82-83, 453-458.

[29] Taylor, P., Wong, L., Radic, Z., Tsigelny, I., Brüggemann, R., Hosea, N., Berman, H. A., 1999. Analysis of cholinesterase inactivation and reactivation by systematic structural modification and enantiomeric selectivity. Chem. Biol. Interact. 119-120, 3-15.

[30] Wong, L., Radic, Z., Brüggemann, J.M., Hosea, N., Berman, H.A., Taylor, P., 2000. Mechanism of oxime reactivation of acetylcholinesterase analyzed by chirality and mutagenesis. Biochemistry 39, 5750-7.

[31] Worek, F., Reiter, G., Eyer, P., Szinicz, L., 2002. Reactivation kinetics of acetylcholinesterase from different species inhibited by highly toxic organophosphates. Arch. Toxicol. 76, 523-529.

[32] Worek, F., Thiermann, H., Szinicz, L., Eyer, P., 2004. Kinetic analysis of interactions between human acetylcholinesterase, structurally different organophosphorus compounds and oximes. Biochem. Pharmacol. 68, 2237-2248.

[33] Worek, F., Eyer, P., Aurbek, N., Szinicz, L., Thiermann, H., 2007a. Recent advances in evaluation of oxime efficacy in nerve agent poisoning by in vitro analysis. Toxicol. Appl. Pharmacol. 219, 226-234.

[34] Worek, F., Aurbek, N., Koller, M., Becker, C., Eyer, P., Thiermann, H., 2007b. Kinetic analysis of reactivation and aging of human acetylcholinesterase inhibited by different phosphoramidates. Biochem. Pharmacol. 73, 1807-1817.

[35] Worek, F., Aurbek, N., Thiermann, H., 2007c. Reactivation of organophosphate-inhibited human AChE by combinations of obidoxime and HI 6 in vitro. J. Appl. Toxicol. in press.