MMSL Military Medical Science Letters

Mil. Med. Sci. Lett. (Voj. Zdrav. Listy) 2012, vol. 81(4), p. 164-170
ISSN 0372-7025
DOI: 10.31482/mmsl.2012.023

ORIGINAL ARTICLE

FROM THE RESEARCH OF CHOLINESTERASE REACTIVATORS TO THE EFFECTIVE THERAPY OF ORGANOPHOSPHATE/NERVE AGENT POISONING

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Received 23rd August 2012.
Revised 19th September 2012.
Published 7th December 2012.

Summary

OBJECTIVES: The aim of this study is to inform about different techniques used to improve treatment of nerve agents intoxication at the Department of Toxicology.

METHODS: Different methods are briefly mentioned and their importance for development of more effective reactivators is discussed.

RESULTS: Basic characterization of nerve agents and therapeutic drugs is necessary after literature survey. Usage of different techniques and properties of various reactivators are studied and, on this basis, the most effective ones are tested in details and proposed for practical use.

CONCLUSIONS: The results described in this study clearly demonstrate that for the development of new and more effective cholinesterase reactivators, a complex approach using different methodical attitudes is necessary.

Key words: acetylcholinesterase; reactivators; methods; therapeutic efficacy; nerve agents

INTRODUCTION

Nerve agents belong to the most toxic organophosphates (OP) in the group of cholinesterase inhibitors. They can be used as chemical warfare agents and, they can be (and were) misused by terrorists, as happened in Matsumoto city (1994) and Tokyo subway (1995). Sarin was used in these cases. Medical protection against their effect (i.e. antidotal treatment) is of prime importance (Bajgar 2004, 2012; Marrs et al. 1996). Moreover, poisoning with OP is commonly reported internationally (Eyer 2003). Therefore, the development of more effective antidotes against intoxication with nerve agents/OP is still necessary.

The basic mechanism of toxic effects induced by nerve agents involves the inhibition of cholinesterases, in particular acetylcholinesterase

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(AChE, EC 3.1.1.7) in the central and peripheral nervous system. The resulting accumulation of acetylcholine at the synaptic junctions desensitizes the cholinegic receptor sites, and symptoms of intoxication are developed (Bajgar 2004; Kassa et al. 2007, 2008; Marrs et al. 1996). The fate of a poisoned organism is very dependent on early administered antidotes. Based on our knowledge of the action mechanism, two therapeutic principles for antidotal treatment are used. The main drugs are anticholinergics which antagonize the effects of accumulated acetylcholine at the cholinergic synapses (also called symptomatic antidotes) and cholinesterase reactivators (oximes) which reactivate inhibited AChE (causal antidotes). Their effects are synergistic. Benzodiazepines are also used to treat convulsions (anticonvulsants) (Bajgar 2004, 2012; Kassa et al. 2007). Treatment of metabolic and ions dysbalance, as well as support of vital functions (heart, ventilation) is necessary. While the use of anticholinergics in the treatment of OP poisoning is unquestioned, some doubts about the use of different reactivators occur in literature. In this paper, our approach and brief results of the research of reactivators is summarized and some examples are shown and discussed.

SOME STUDIES DEALING WITH AChE REACTIVATORS

At the Department of Toxicology, the effects of nerve agents are studied with the aim to improve medical protection against these compounds. It is based on good knowledge of pharmacodynamics of nerve agents as well as reactivators. At the very beginning, literature survey and other informations are necessary (Bajgar 2004; Kassa et al. 2007; Voicu et al. 2010); after this stage, own research follows. For this purpose, different approaches and techniques are used as demonstrated below: The scheme of examination was described (Kassa et al. 2007) previously and it contains the following steps: after synthesis, screening of AChE inhibition/reactivation (at two oxime concentrations) of selected nerve agents is performed (up to now, more than 1000 compounds were tested). The next step is the study of inhibition/reactivation at a limited concentration rank (hundreds compounds). Kinetics (inhibition/reactivation) with 4 nerve agents and a large scale of reactivator concentration is the next step; about 50 compounds are involved. *In vivo* testing (toxicity in mice, rats and guinea pigs; reactivation; therapeutic index and behavior) is following (about 20 compounds). For the very promising oxime (6), further studies (plasma concentration, penetration the BBB and others) are performed.

Inhibition/reactivation potency of the nerve agent/reactivator to AChE can be used to characterize the toxic effects (determination of cholinesterase activities). According to the procedure and laboratory instrumentation, the most common methods of cholinesterase determination are as follows: manometric (one of the oldest methods), electrometrical, titrimetric, colorimetric detection of the unhydrolyzed substrate, measurement by the change of pH using an indicator, spectrophotometric, fluorimetric, radiometric, calorimetric, polarographic, enzymatic, and others. These methods are also suitable for the detection of cholinesterase inhibitors using biosensors or immunochemical assay for detection of chemical warfare agents. A more detailed review dealing with the methods of cholinesterase determination including literature sources was given previously (Bajgar 2004, 2012; Jun et al. 2009). A very sensitive and commonly used method for cholinesterase determination was described by Ellman et al. (1961).

At our Department, cholinesterase activity is determined mostly by spectrophotometric (Ellman) and titrimetric methods. A flow injection analysis for determination of cholinesterase activities in a biological material is not a very usual example of its use (Cabal et al. 2010) and it is also employed at our Department. Moreover, this method can be useful for continual monitoring of cholinesterase activity *in vivo*. Thus, determination of cholinesterase activities in different biological materials is a basic and necessary method for the studies dealing with improvement of therapeutic efficacy.

Modeling of the reactivator´s effect *in vitro* is a further approach (Bajgar 2004; Kuca et al. 2010; Musilek et al. 2010a) including inhibition and reactivation studies *in vitro* as the basic information for comparison of inhibition potency (in relationship with toxicity) and toxicity assessment. The research is supported by reactivation experiments including docking studies to achieve better reactivation or other effects (Musilek et al. 2010b). The results are contributing to the search for new structures with better reactivation effectiveness. A hypothetic example of AChE reactivation is shown in Fig. 1. The obtained results can be of a different character and the most promised reactivators are further tested. Moreover, this approach is not limited to the antidotal effect against nerve agents but it is contributing for the search for new drugs against Myasthenia gravis or Alzheimer´s disease (Musilek et al. 2010a).

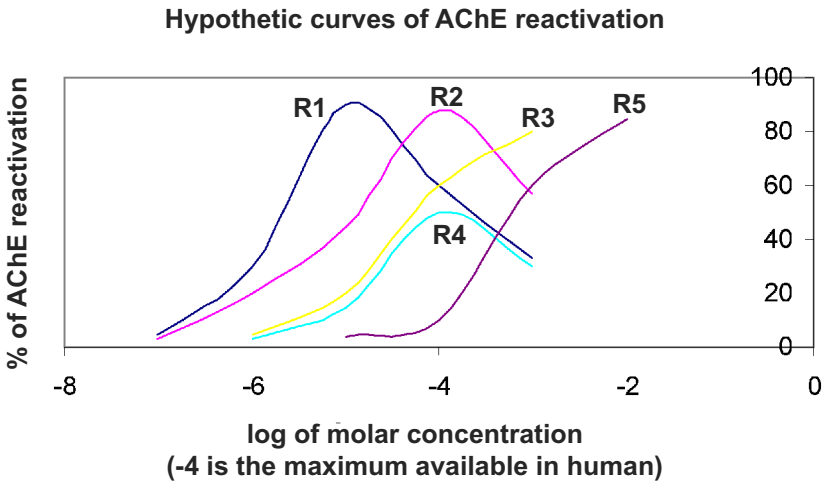

Figure 1. Hypothetic curves of AChE reactivation by reactivators with different effectiveness *in vitro*. R1 – oxime with higher reactivation efficacy than R2; however, final effect will be dependent on their toxicity: if the toxicity of R1 is high, then R2 will be more prospective. R3, R4 are oximes practically with the same reactivation efficacy in the concentration 10^{-4} M; however, for R4, reactivation decreases in higher concentration and thus, R3 is more suitable for further study. R5 – oxime with high efficacy but the concentration for this effect is too high for achievement in biological material following administration *in vivo*.

The necessary basic information *in vivo* is a simple characterization of the compound by toxicity. Some data are shown in Table 1, demonstrating toxicities of different nerve agents during 20 years. Using toxicity data for different species *in vivo* and inhibition potency *in vitro* can lead to assessment of toxicity of nerve agents to men (Bajgar et al. 2009). For testing effectiveness of prophylaxis/treatment, the best choice is determination of toxicity before and after all therapeutic or prophylactic intervencies (as the change of toxicity); the ratio of LD50 after therapeutic or prophylactic intervention/LD50 without intervention is described as therapeutic or prophylactic index (TI or PI). An example of prophylactic efficacy is shown in Table 2.

Table 1. Toxicities (i.m.) for different nerve agents in mice expressed as LD_{50} (µg/kg, i.m.) in period 1992-2010 years; means and variations (min-max)

NERVE AGENT	mice (1996-2010)	rats (1992-2010)
VX	25 (16-38)	15 (8-28)
GV	30.5 (18-65)	17 (9-31)
soman	111.9 (60-150)	78.7 (51-102)
sarin	170.3 (120-241)	129.7 (100-180)
cyclosarin	190 (155-232)	80 (62-103)
tabun	275 (211-301)	168 (102-201)

For the treatment, reactivation efficacy *in vivo* can be provided too, either for administration of the reactivator alone or for their combinations (Bajgar et al. 2010; Kassa and Cabal 1999; Kassa et al. 2009) (Fig. 2). The effectiveness of combination of two reactivators indicates that the effect is not simply summation but more probably potentiation. These results with two reactivators demonstrated that the effect of reactivator´s combination could be a solution in search for a universal reactivator. Therapeutic efficacy can be evaluated by the method of isoboles (Fig. 3). It can be used for more precise determination of therapeutic interaction such as synergistic or summation effects but this method is expensive and requires a large amount of experimental animals.

Table 2. Comparison of prophylactic efficacy (± SD) of different prophylactics (expressed as prophylactic index) against some nerve agents in rat. Summarization of different results (Bajgar 2004; Kassa et al. 2007). Equine BuChE was kind gift of Dr. B. Doctor, WRAIR, USA.

proPHylaCtic Combination	VX	sarin	soman	RVX	tabun
PANPAL	15,9±2.44	3,4±0.51	2,0±0.55	10,8±2.12	2,2±0.67
TRANSANT	1,0±0.23	0,95±0.21	1,0±0.23	1,01±0.38	1,1±0.43
PANPAL+TRANSANT	18,7±2.3	5,8±1.03	4,1±1.04	15,6±2.12	6,8±2.12
EqBuChE	5,5±1.0	4,3±0.97	3,9±1.00	5,1±1.32	4,8±1.33
PYRIDOSTIGMINE (alone)	1,5±0.88	1,8±0.67	1,3±0.78	1,4±0.76	2,0±0.45

For further studies of nerve agents action mechanism *in vivo*, AChE inhibition/reactivation in different tissues (mostly, the rat or human brain homogenate in distilled water 1:10 is used as a source of AChE) is determined. Homogenates of different tissues are used for biochemical AChE determination, not allowing to detect changes in different structures including the brain and its areas. Quantitative histochemical determination of AChE activity allows fine differentiation for various brain structures (Fig. 4) (Bajgar et al. 2007a,b; Hajek et al. 2010). Although AChE inhibition detected biochemically showed a similar degree of inhibition, quantitative histochemistry of AChE demonstrated that inhibition effects are different for various inhibitors (e.g. VX, and Russian VX). The results showed that AChE activity/inhibition detected by quantitative histochemistry is more informative than determination of AChE activity/inhibition in homogenates of the brain parts using biochemical methods. Nevertheless, the results of both methods (histo- and biochemical) correlate very well (Bajgar et al. 2007a,b; Hajek et al. 2010).

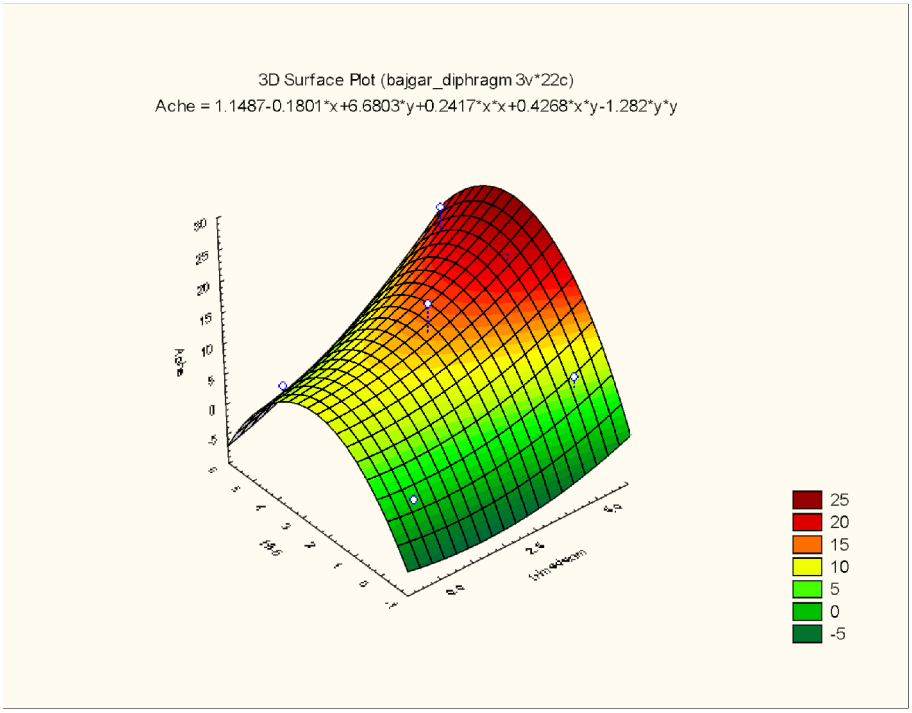

Figure 2. Reactivation of AChE in the diaphragm following intoxication with tabun (300 µg/kg, i.m.) and treatment with atropine and combinations of HI-6 and trimedoxime. The data from Bajgar et al. (2010) evaluated by 3D transformation. axes: x – dose of HI-6; y - % of AChE reactivation; z – dose of trimedoxime.

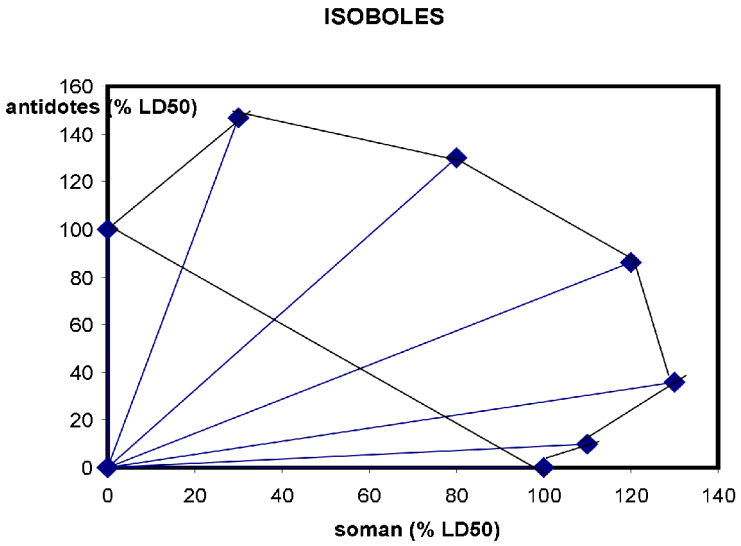

Figure 3. Method of isoboles. Combined lethal effect of soman in mice. Combination of soman (i.m.) and antidotes (atropin and trimedoxime, i.m., 2 minutes after the intoxication) in mice. 100% of LD_{50} = 148.8 mg/kg.

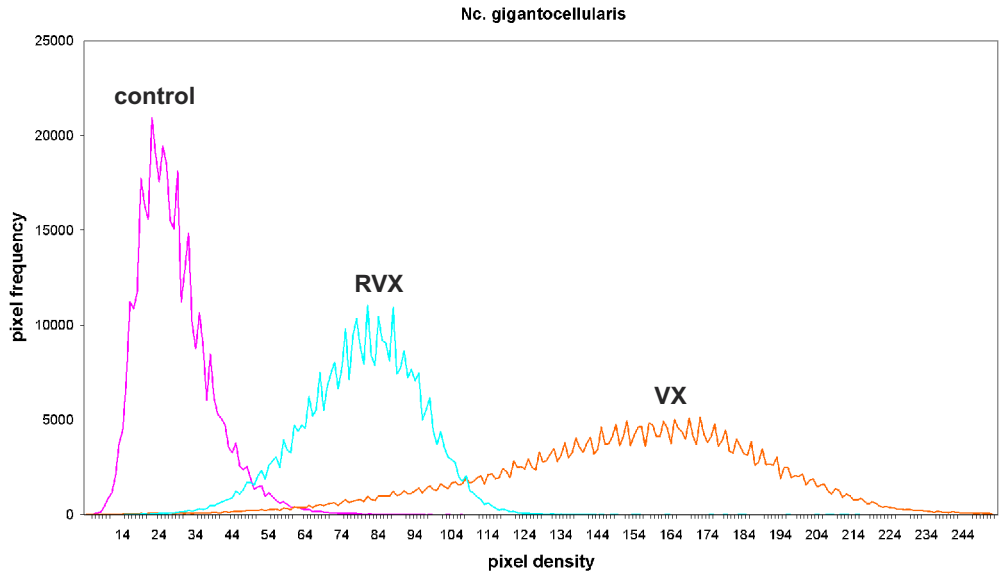

Figure 4. Quantitative histochemistry of AChE detection in the reticular formation of the rat brain. The effects of VX and Russian VX in comparison with control. Methodical details were described previously (Bajgar et al. 2007b). Quantitative evaluation was kindly submitted by Dr. P. Hajek, Department of Anatomy, Medical Faculty, Charles University in Hradec Kralove.

Blood and tissue levels of nerve agents and antidotes are a necessary part of these studies (Karasova et al. 2010, 2011). It is used for verification of real oxime concentration in the examined tissues and it is also used for comparison of reactivation experiments: achieved concentration needs to be as high as possible for real AChE reactivation determined in previous experiments.

Behavioral studies show the effect of agents and their antidotes on different parameters of behavioral activity; the results were published for different reactivators (Kassa et al. 2007, 2009). Mechanisms other than the cholinergic ones (oxidoreduction potential etc.) are also studied (Pohanka et al. 2011). There are other methods *in vitro* and *in vivo* for the study of reactivator´s effect (Fisar et al. 2011; Kassa and Cabal 1999; Soukup et al. 2010).

All these studies are performed with the strategy to obtain more effective reactivators by improving their penetration through the blood brain barrier as well as to increase their reactivating efficacy. It can be obtained by their structural modifications and combination of the oxime structure with others (e.g. sugar).

CONCLUSIONS

Using these methodical approaches, real means for the treatment and prophylaxis against nerve agents were studied, developed and introduced into the Czech Army. They are as follows:

-reactivators for medical treatment – methoxime (RENOL) and HI-6 (ANTIVA);

-prophylactics – PANPAL (tablets containing combination of pyridostigmine, and benactyzine with trihexyphenidyle) and TRANSANT (plaster for transdermal administration of HI-6);

-some other drugs and approaches are under experimental studies - e.g. huperzine A (Bajgar et al. 2007a), combinations of reactivators (Bajgar et al. 2010; Kassa et al. 2010), and new reactivators (Kassa et al. 2007, 2008, 2009; Kuca et al. 2010; Musilek et al. 2010a,b).

-some results and conclusions are summarized in the book published this year (Bajgar 2012)

This illustrative review is not exhaustive and new approaches and methods are used or are under development.

ACKNOWLEDGMENT

The study was supported by the grant of Ministry of Defence, „A long term organization development plan 1011“.

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