Journal of Enzyme Inhibition and Medicinal Chemistry, June 2009; 24(3): 680–683

In vitro reactivation of trichlorfon-inhibited butyrylcholinesterase using HI-6, obidoxime, pralidoxime and K048

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(*Received 11 March 2008; accepted 19 May 2008*)

Abstract
Trichlorfon is a specific inhibitor of cholinesterases. It was typically used as an insecticide; however, trichlorfon was described as useful for symptomatic treatment of Alzheimer's disease some years ago. The presented study is aimed at reactivation of trichlorfon-inhibited butyrylcholinesterase since this enzyme play an important role in Alzheimer's disease as deputy for acetylcholinesterase and furthermore it could be applied as a scavenger in case of overdosing. We used *in vitro* reactivation test for considering only reactivation efficacy of butyrylcholinesterase that is inhibited by trichlorfon and not reactivation of butyrylcholinesterase inhibited by trichlorfon metabolic products. Four reactivators were used: HI-6, pralidoxime, obidoxime, and K048. Although all of the reactivators seem to be effective at 1 mM concentration, a lower concentration was not able ensure sufficient reactivation. There was also an observed lowering of reactivation efficacy when butyrylcholinesterase was exposed to trichlorfon for a longer time interval.

Keywords: *metrifonate, chlorophos, Alzheimer's disease, reactivation, oxime, butyrylcholinesterase, trichlorfon*

Introduction

Trichlorfon is an organophosphonate pesticide commercialized under lots of trade names including the well known metrifonate and chlorophos. It is an inhibitor of important enzyme in neurosynapses: acetylcholinesterase (AChE; EC 3.1.1.7). According to the IUPAC, the correct name of this compound is following: (RS)-2,2,2-trichloro-1-(dimethoxyphosphinoyl)ethanol. Common application of trichlorfon is treatment of vegetable, fruit, and forest and so on when damaging by insect occurs. Trichlorfon is also well known medicament for effective suppression of cognitive and behavioral symptoms of Alzheimer's disease [1–3]. The efficacy and low side effects of trichlorfon were proved on a total of 408 patients [4]; on the other side, trichlorfon could induce aneuploidy in male mouse germ cells [5]. After intake by organism, trichlorfon is spontaneously transformed into 2,2- dimethyl dichlorovinyl phosphate that is more affine to serine in AChE active centre than the original trichlorfon [6,7].

Reactivation of cholinesterases inhibited by organophosphates and organophosphonates is needed when intoxication occurs; reactivators used for reactivation purposes are compounds with oxime functional group able to dissociate phosphate or phosphonate from cholinesterase [8]. Because of evolutionary rigid structure of cholinesterases, the similar reactivation efficacy is widely expected for cholinesterases from various organisms [9]. Wide group of reactivators is commonly known, especially obidoxime, pralidoxime, and HI-6 are the commercialized ones; however, new reactivators are referred, too [10–16].

The presented study follows the efficacy of butyrylcholinesterase (BChE; EC 3.1.1.8) reactivation using currently available reactivators. BChE plays

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ISSN 1475-6366 print/ISSN 1475-6374 online © 2009 Informa UK Ltd.
DOI: 10.1080/14756360802328315

substitute role in organism [17], especially when AChE is inhibited by low dose of trichlorfon for treatment purposes. Previously described photometric method was chosen as a convenient tool for reactivation monitoring [18]. The *in vitro* method was chosen for possibility of monitoring reactivation of only trichlorfon and not its metabolic products since in some cases of acute intoxication such as overdosing in Alzheimer's disease treatment could be trichlorfon harmful itself.

Material and methods

Chemicals

Trichlorfon in analytical purity was purchased from Labor Dr. Ehrenstorfer-Schafers (Augsburg, Germany). Acetylthiocholine chloride (ATChCl), 5,5′-dithiobis (2-nitrobenzoic acid) (DTBN), and BChE from human serum were purchased from Sigma-Aldrich (Czech Republic branch). Pralidoxime (1-methyl-2-hydroxyiminomethylpyridinium chloride), obidoxime (1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxapropane dichloride), HI-6 (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dichloride) and K048 (1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium)-butane dibromide) were previously synthesized accordingly to references [19–22]. Used reactivators are presented in Figure 1. All substances were diluted by phosphate buffered saline (PBS).

Measuring format

Plates with 96- wells (Gama, Ceske Budejovice, Czech Republic) were used throughout all experiments. BChE was adjusted up 10 nkat/mL by PBS dilution. 10 μL of BChE solution and 10 μL of trichlorfon (or 10 μL of PBS as negative control) solution (0.5 mM) were mixed together within one well and let incubate for 0.5 h or for 1 h. Then 1 mM ATChCl with DTBN 0.4 mg/mL (in PBS) was injected in amount 40 μL per well. Immediately after that, 10 μL of given concentration of reactivator (or PBS) was injected per well and the absorbance shift at 412 nm was followed using MRX device (Dynatech Laboratories, Chantilly, VA, USA). Absorbance was measured after 15 min. Obtained experimental data were processed according to the following equation:

$$R = \frac{A_r - A_b}{A_0 - A_b} \times 100(\%) \quad (1)$$

The percent of reactivation (R) is expressed as function of absorbances measured in wells with reactivated BChE (A_r), intact BChE (A_0). Absorbances A_r and A_0 are compared to the blank (reaction mixture: 1 mM ATChCl with DTBN 0.4 mg/mL in PBS) absorbance - A_b.

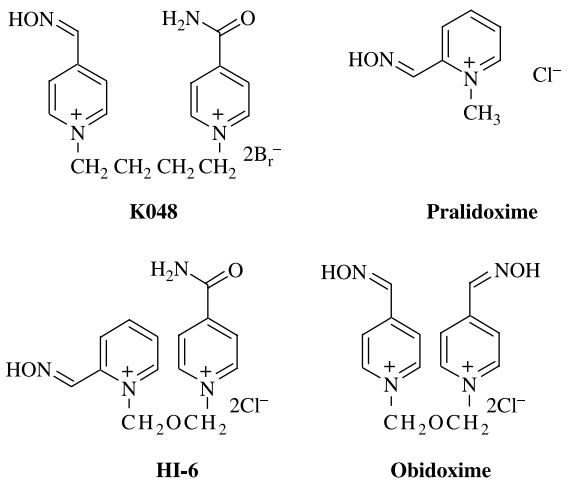

Figure 1. Reactivators used.

Results and discussion

Experiment was designed as an *in vitro* study for reactivation of BChE inhibited by trichlorfon using photometric microplate assay using previously proposed protocol [18]. Reactivation *in vitro* does not typically provide a complex data since several effects in body such as partial metabolizing could influence achieved results. The presented study is targeted as *in vitro* study because of above mentioned trichlorfon metabolizing so followed reactivation of cholinesterases in tissues or organisms is typically concerning to 2,2- dimethyl dichlorovinyl phosphate when *in vivo* experiment is chosen. Our study is based only on reactivation of trichlorfon inhibited BChE because BChE is commonly useful in way of scavenger. Furthermore, it plays a deputy role in organism when Alzheimer's disease is treated by trichlorfon [23].

Inhibition of BChE was realized using four potentially effective reactivators: HI-6, obidoxime and pralidoxime are reactivators that were used in some armies as therapeutic for individuals poisoned by nerve agents; K048 is previously synthesized reactivator with properties similar to the above mentioned ones. The achieved data are digestedly summarized in Figure 2 for reactivators application within half an hour after BChE inhibition and in Figure 3 for reactivators application within one hour.

One common effect was obvious – the concentration of reactivator should reach 1 mM for effective reactivation (above 50%). Only low reactivation was observed for reactivators in concentration 0.1 mM and 0.01 mM. Efficacy of reactivation strongly decreases (R decrease about 10% for all of reactivators in concentration 1 mM) when time interval of trichlorfon and BChE preincubation is prolonged from half an hour to one hour. These facts respond to expectancies

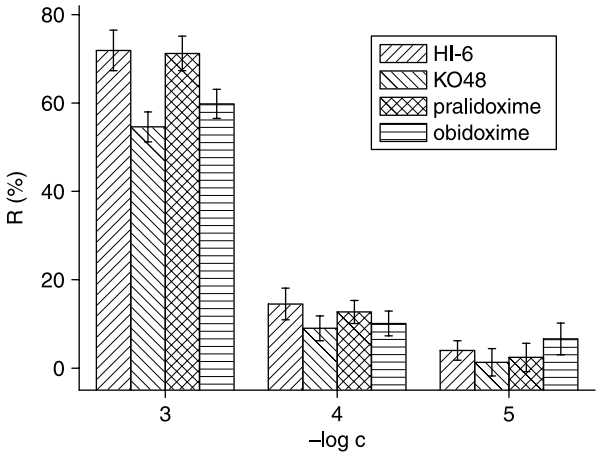

Figure 2. Semilogarithmic expression of reactivation efficacy. Percent of reactivation (*R*) vs. logarithm of reactivator molar concentration (final on plate). Reactivation was realized after 0.5 h incubation of trichlorfon with butyrylcholinesterase. Error bars indicate standard deviation (n = 4).

especially when we considered spontaneous dealkylation (aging) of trichlorfon bound in enzyme active centre and no chance to reactive dealkyled organophosphate from this centre [24,25]. The difference between half an hour and one hour preincubation before reactivation application is significant on probability level approx. 0.2.

The highest efficacy of trichlorfon inhibited BChE reactivation proved two reactivators: HI-6 and pralidoxime in concentration 1 mM. The high reactivation by HI-6 was quite surprising when we consider strong competitive effect between this reactivator and substrate not only for AChE but also for BChE [26]. We should consider reactivator concentration 1 mM as the lower limit since all reactivators in concentration 0.1 mM and lower provided efficacy under 20%. The increase of reactivator concentration strongly above 1 mM is not convenient due to starting of intoxication symptoms resulting in cholinergic crisis similar to that when individuals intoxicated by organophosphate.

An intriguing conclusion could be realized if the efficacy (percent of reactivation *R*) of HI-6 and pralidoxime is compared with obidoxime and K048. Though pralidoxime and HI-6 are monoquaternary reactivators, both are more effective for reactivation purposes than bisquaternary obidoxime and monoquaternary K048. It seems that more important for reactivation of BChE inhibited by trichlorfon purposes is position of oxime group (HI-6 and pralidoxime have oxime in position ortho in comparison with position para for obidoxime and K048) rather than monoquaternary or bisquaternary structure.

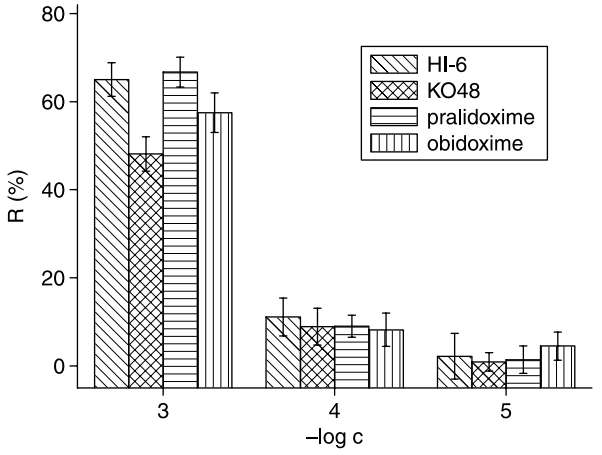

Figure 3. Semilogarithmic expression of reactivation efficacy. The meaning of symbols is the same as in Figure 2. Data were obtained for reactivation after 1 h of incubation of butyrylcholinesterase with trichlorfon.

Conclusion

The efficacy of trichlorfon-inhibited BChE treatment was followed in this study using a photometric microplate assay. The *in vitro* model was chosen to avoid the partial metabolism of trichlorfon in the body or tissues. So that the reactivation efficacy is selective for only trichlorfon.

Acknowledgements

This work was supported by the Ministry of Defence of the Czech Republic, Grant No. FVZ0000604.

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

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