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**RESEARCH ARTICLE**

# Changes of rat plasma total low molecular weight antioxidant level after tabun exposure and consequent treatment by acetylcholinesterase reactivators

Miroslav Pohanka[1,2], Jana Zdarova Karasova[2], Kamil Musilek[2], Kamil Kuca[1,2], Young-Sik Jung[3], and Jiri Kassa[2]

*[1]Centre of Advanced Studies, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic, [2]Department of Toxicology, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic, and [3]Medicinal Science Division, Korea Research Institute of Chemical Technology, Yusong, Taejon, Korea*

**Abstract**

These experiments were performed on a rat model. The rats were divided into eight groups and consequently exposed to either a saline solution (control), atropine or a combination of atropine and tabun. The reactivation efficacy of the oximes was estimated on the rats exposed to tabun, atropine and a reactivator of AChE. The oximes HI-6, obidoxime, trimedoxime, K203 and KR-22836 were used as representative compounds of commonly available and new AChE reactivators. Besides the positive effect of the administered reactivators on blood AChE activity, the sizable modulation of low molecular weight antioxidant (LMWA) levels was also determined. The LMWA levels in the the animals treated with the oxime reactivators were decreased in comparison with the animals treated by atropine alone. It was found that the levels of LMWA returned to the level found in the control animals when either trimedoxime, K203 or KR-22836 were administered. The principle of oxime reactivator function and a novel insight into AChE activity regulation and oxidative stress is discussed.

**Keywords:** Acetylcholinesterase, nerve agent, antioxidant, reactivator, cholinergic antiinflammatory pathway, tabun, oxime

## Introduction

The acute toxicity of organophosphorous pesticides, nerve agents and some natural toxins and drugs is based on the inhibition of acetylcholinesterase (AChE; EC 3.1.1.7). However, other important enzymes are sensitive to the inhibitory effects of the above mentioned compounds. Butyrylcholinesterase, trypsin, acetylCoA hydrolase, succinylCoA hydrolase, phospholipase A2 and other enzymes can also be introduced. The physiological function of AChE is based on the termination of neurotransmission through the neuronal junction via hydrolysis of the neurotransmitter acetylcholine [1]. Another function of AChE is the cholinergic anti-inflammatory pathway associated with the nervus vagus [2]. In macrophages, acetylcholine activates the α7 nicotinic acetylcholine receptor (nAChR). A stimulated nAChR prevents the nuclear factor κB initiating production of TNF as well as the high mobility group box 1 [3,4].

AChE can be inhibited by many compounds. Some toxins such as aflatoxins are able to deform the active gorge of AChE and inhibit it non-competitively. Competitive inhibition is based on the reversible binding of toxins or drugs such as HI-6 to the peripheral anionic site of AChE. The most serious effects are as a result of the inhibition of the esteratic site by highly toxic compounds such as the organophosphorous pesticides and nerve agents [5–7].

The AChE inhibitors (e.g. malathion) can also be causative agents of oxidative disorder and the stimulation of glutathione-based antioxidative pathway can prevent oxidative stress-induced impairment after such

*Address for Correspondence*: Miroslav Pohanka, Faculty of Military Health Sciences, University of Defence, Trebesska 1575, 50001 Hradec Kralove, Czech Republic. E-mail: rau@atlas.cz or pohanka@pmfhk.cz

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intoxication [8]. The antioxidative defence mechanism sharply increases and counteracts the impairment of oxidative balance as indicated by the important marker, malondialdehyde [9]. The total level of antioxidants can also be shifted when drugs reactivating inhibited AChE are administered [10].

This study was carried out as an attempt to estimate the effects of the currently available as well as some new AChE reactivators suitable for the treatment of intoxication by a highly toxic anticholinergic compound, namely tabun. Following the important findings from our previous research [10], we have designed some new experiments based on equimolar rather than therapeutic doses. Though the effect of drugs suitable for reactivation of AChE inhibited by organophosphates or organophosphonates is generally known [5], this research is concerned with the understanding of oxidative stress modulation.

## Experimental

### Drug synthesis and purity examination

The nerve agent tabun (*O*-ethyl-*N,N*-dimethylphosphoramidocyanidate) was obtained from Military Technical Institute of Protection (Brno, Czech Republic) in compliance with permission for the handling of chemical warfare agents. Atropine hydrogen sulphate was purchased in pure form from Sigma-Aldrich (Prague, Czech Republic). The AChE reactivators: HI-6 dichloride, obidoxime, trimedoxime and K203 were synthesised at the Department of Toxicology, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic [11] and the Medicinal Science Division, Korea Research Institute of Chemical Technology, Taejon, Korea. The purity of the prepared reactivators was confirmed by HPLC/MS assay. The structures of the oxime reactivators that were used are depicted in Figure 1.

### Animals and experimental intoxication

Male wistar rats weighing 180–200 g were used for the experiments. The animals were purchased from Anlab (Prague) and processed shortly after an obligatory quaranteen. The animals were kept in an air conditioned room (22 ± 2°C) with controlled humidity (50 ± 10%) and light (7 am to 7 pm) in the Central Vivarium of the Faculty of Military Health Sciences, University of Defence, Hradec Kralove. Food and water were provided *ad libitum*. Permision to carry out these experiments was granted by the acredited ethical committee who supervised any manipulations with animals as well as the consequent experiments.

The animals were divided into eight groups with six animals per group. The animals were exposed intramuscularly (im) by saline solution and/or tabun ($LD_{50}$; 180 µg per kg of body weight – kg bwt) and atropine (21 mg/kg bwt). The reactivators HI-6, obidoxime, trimedoxime, K203 or KR-22836 were administered five minutes prior to tabun at a dose of 60 µmol/kg bwt. The system of dosing in individual groups was as follows:

Control group was exposed to only saline solution.
Atropine and saline solution.
Atropine five minutes prior to tabun.
Atropine and HI-6 prior to tabun.
Atropine and obidoxime prior to tabun.
Atropine and trimedoxime prior to tabun.
Atropine and K203 prior to tabun.
Atropine and KR-22836 prior to tabun.

The rats were sacrificed under anaesthesia in $CO_2$ half an hour after receiving the tabun poison. Blood was collected in heparinised tubes from the carotid artery. Plasma was prepared by centrifugation (3000g for 15 minutes at 15°C). The blood and plasma samples were processed immediately due to the poor stability following storage.

### Biochemical evaluation of the AChE activity

The activity of blood AChE was carried out by an adapted Ellman's method [10,12]. Blood was haemolysed in 0.02 M Tris buffer (pH 7.6) by mixing the blood with buffer in a ratio of 1:20. The AChE activity was measured shortly after haemolysis. An aliquot of 200 µL of the blood lysate was mixed with 800 µL of a solution of 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) 0.4 mg/ml and 1 mM acetylthiocholine chloride (ATChCl). A suspension with 200 µL physiological solution instead of the blood lysate was used as a blank. The absorbance was measured at 412 nm after 5 minutes of incubation.

### Cyclic voltammetry

The total level of the low molecular weight antioxidants (LMWA) in plasma was measured by cyclic voltammetry. An aliquot of 20 µL of plasma was spread over the voltammetric strip including the graphite working, Ag/AgCl reference and graphite auxiliary electrodes (BVT, Brno, Czech Republic). Cyclic voltametry was performed by an EmStat device (PalmSens, Houten, The Netherlands). The range of applied voltage was –0.1 to 1.1 V and the scan rate was adjusted to 50 mV/s. The antioxidants appeared as a wave in the anodic range [10,14–16].

## Results

The rats were exposed to tabun and one of the five oximes in combination with atropine. Our previous experiments were designed to administer the drug dose calculated from its toxicity as a therapeutical dose corresponding to 5% of the $LD_{50}$ [10]. The present study aimed to evaluate two novel drugs: K203 and its fluorinated analogue KR-22836 and compare them with HI-6, obidoxime, and trimedoxime. In contrast to the previous experiments, equimolar doses of oximes were administered into the animals. All the drugs were selected from previous studies as being potent AChE reactivators *in vitro*; obidoxime, trimedoxime and HI-6 are currently available in many countries including NATO members [13–15]. Their potency to reactivate AChE was evaluated by *in vitro* studies and seemed to be sufficient

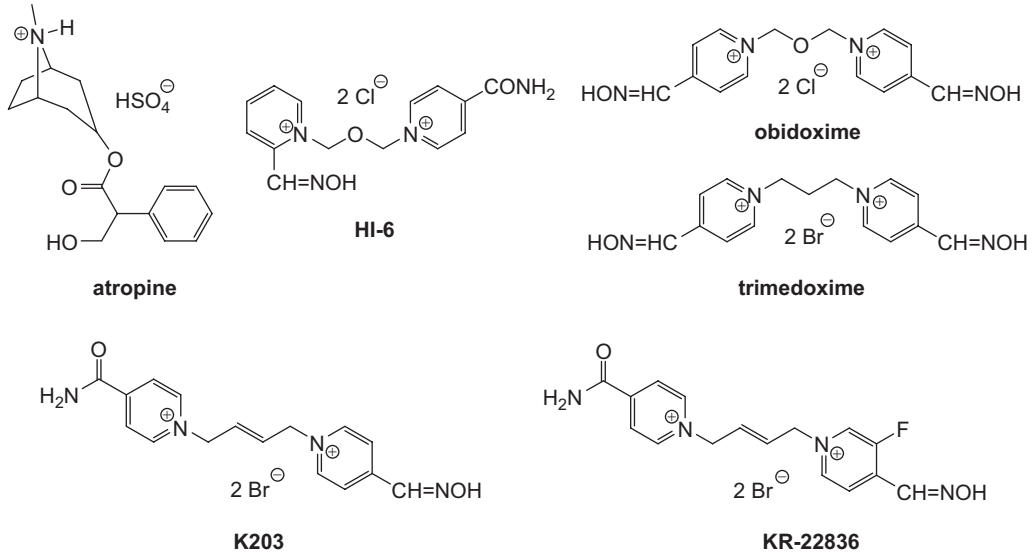


Figure 1. The structures of the drugs used in the experiments.

[16]. On the other hand, their efficacy is limited by the safe dosage and this constrains their reactivation efficacy [17]. Hence, the measured AChE activity might appear higher than the real AChE activity due to oximolysis [18]. The efficacy of the reactivators used to recover the blood AChE activity is presented in Figure 2A. The initial activity of AChE in the blood was found to be 19.5 ± 3.5 μkat/mL. One $LD_{50}$ of tabun caused a strong decrease of AChE activity to 6.7 ± 0.5 μkat/mL in thirty minutes, conforming to a residual activity of 34%. Tonic-clonic seizures can be a typical manifestation of this level of intoxication [19] and these convulsions were observed after tabun exposure. The administration of reactivators increased the activity from 6.7 to 7.4–11.1 μkat/mL depending on which oxime was used. The best reactivation potency was found to be for obidoxime and K203. The recovery of AChE activity was not significant (ANOVA with Scheffe test, $P \leq 0.05$, statistical evaluation using Origin 8; Origin Lab Corporation, Northampton, MA, USA) for HI-6. The other reactivators provided a significant reactivation, although the activity wasn't completely restored. The blood samples from the treated animals had a significantly lowered AChE activity, when compared to the intact animals.

The LMWA found in plasma were assayed as a second important marker of overall body shape and the experimental data are summarised in Figure 2B. The changes in the LMWA were compared to the blood AChE activity. Administration of atropine induced an increase of LMWA four times the level compared with the control group. On the other hand, no significant differences were found between the animals exposed to atropine only and the animals exposed to both tabun and atropine. A surprising effect was provided by the reactivators that significantly decreased the LMWA levels in comparison with the samples exposed to atropine alone. A comparison of LMWA levels in plasma from animals treated by the reactivators and the control group showed significant differences for HI-6 and obidoxime treated plasmas, while the other oximes did not show a significant difference in the plasma LMWA level compared to the control values.

The correlation of LMWA levels with increased AChE activity has not provided valuable data. The correlation was slightly negative ($R = -0.19$) i.e. the oxime reactivators with a lower reactivation efficacy can be expected to increase the LMWA levels, however, this hypothesis was not found to be significant.

## Discussion

The LMWA levels in plasma and blood cells provide an organism with protection against various pathological conditions. A strong decrease in antioxidant levels is accompanied by deterioration and pathological conditions such as cancer or AIDS [20]. An increase of LMWA is associated with stress that may arise in non-pathological conditions [21]. Healthy organisms are able to protect themselves even when they are exposed to high doses of toxic compounds such as sulphur mustard [13]. Oxidative stress arises when an organism is insufficiently protected and this can occur shortly after a toxicity burden [22,23]. Stressful conditions can facilitate the penetration of an inhibitor through the blood-brain barrier [24]. A protective mechanism against stress would be provided by the cholinergic anti-inflammatory pathway [25–26].

There is a question related to the reactivator efficacy. The reactivation of blood AChE is limited. The effect of reactivator in CNS is even lower than the effect in blood due to the poor penetration of oxime through the blood-brain barrier being usually less than 10% [27]. The therapeutic effect of the reactivator should be considered as a sum of the partial impacts on an organism resulting in the final benefit. The most potent reactivators are considered to be the compounds with minimal toxicity, good reactivating efficacy and pharmacokinetics [28]. On the other hand, another positive effect would be the modulation of the cholinergic-anti-inflammatory pathway with

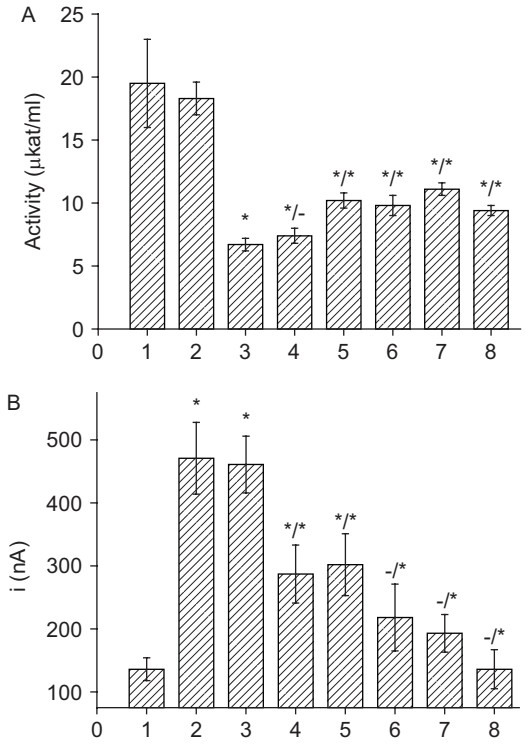


Figure 2. The influence of oximes on (A) AChE activity and (B) LMWA levels. The samples were obtained from (numbered according bar): 1 – controls (intact rats); 2 – rats treated by atropine; 3 – rats exposed to tabun and treated by atropine; 4–8 rats exposed to tabun and treated by atropine combined with HI-6 dichloride (4), obidoxime (5), trimedoxime (6), K203 (7), KR-22836 (8). Asterisks indicate significance (ANOVA with Scheffe test, $p \leq 0.05$) against control (bar 1). When a fraction is presented the asterisk in the numerator indicates significance against the control and an asterisk in the denominator indicates significance against the tabun exposed rats (bar 3). A stroke indicates no significance. The error bars indicate the standard error of mean (SEM) for $n = 8$.

an impact on the overall shape of the body through the modulation of TNF. Inflammation could be accompanied by a blood-brain barrier failure and the subsequent increased penetration of toxic compounds [24]. The effect of antioxidants in AChE associated diseases is still unclear [29]. Reactivation of blood AChE can probably restore proper function of the cholinergic anti-inflammatory pathway since it is assisted by blood cells including the innate immunity cells [26].

The undisputed positive effect of reactivators on the shape of intoxicated individuals has been proven previously. The benefit on behavioural changes in intoxicated versus treated animals in functional observational battery experiments has been observed [30]. In the data presented and in the experiment referred to, the oxime reactivator impact on the stressful conditions of the organism with regard to the drug pathways were considered [10]. On the other hand, polar compounds were better able to penetrate the blood brain barrier under stressful conditions. A significant decrease of LMWA in animals intoxicated by tabun and treated by reactivators compared to the animals treated by atropine only pointed to reduced stress. The changes in plasma LMWA seem to be distinct from the changes in AChE activity when considering the individual oxime reactivators. The oxime reactivators were also assayed by cyclic voltammetry and a ferric reducing antioxidant power assay *in vitro* in an unpublished experiment. This experiment confirmed that oxime reactivators can participate as weak antioxidants. This was also shown for amido-carbonyl oxime derivates by Ozen et al. [31]. However, the direct antioxidant effect of oxime reactivators is not detectable *in vivo* due to their low final concentration in plasma. The advantage of having a low *in vivo* plasma concentration is that there is no interference with any endogenous LMWA changes within the experiments.

The tested oxime reactivators were found to be of two types. HI-6 and obidoxime provided a significant difference to the control animals with regard to the LMWA plasma level. In contrast, trimedoxime, K203 and KR-22836 did not cause a significant change in the LMWA levels. The major structural difference between the HI-6 and obidoxime group compared to the trimedoxime, K203 and KR-22836 group was that the second group did not contain an ether group in the link between the pyridinium moieties. The effect of the amount of oxime functional groups in the reactivator molecule on stress has not been proved. We do not yet understand these phenomenons and will require further investigation.

Atropine is commonly considered to be an effective antidote reducing intoxication manifestation as caused by nerve agents. Dejan et al. extensively reviewed the impact of AChE inhibitors on the launching of reactive oxygen species as well as iso- and neuroprostane generation [32]. The application of atropine with *N*-methyl-D-aspartate receptor antagonists has been shown to prevent oxidative stress [32]. Here, atropine itself caused a significant increase in plasma LMWA even without intoxication with nerve agents. The ambivalent action of atropine was in accordance with the previous results; on the one hand there was a reduction in the impact of the nerve agents and on the other stress was triggered as represented by increased levels of LMWA. The complete mechanism of this ambivalent action of atropine has still not been fully described. Similarly, the investigation of cholinesterase inhibition is not completely explained and extensive research continues [33–35].

## Conclusions

Two effects have been observed on experimental animals exposed to tabun and treated by atropine only or by atropine together with AChE reactivators. The first effect was a partial restoration of AChE activity on account of its reactivation. The second effect was the impact of intoxication and treatment by oxime reactivators on the low molecular weight antioxidant plasma levels. Our study presumes that the beneficial effect of oxime reactivators is not only based on AChE reactivation, but also on the more complicated regulation of processes including antioxidant power balance.

## Declaration of interest

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