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## ORIGINAL ARTICLE

# Inhibition of blood cholinesterases by nerve agents *in vitro*

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**Summary**
The bimolecular rate constants ($k_a$) of inhibition were determined for cholinesterase in rat blood *in vivo* and three nerve agents *in vitro*. They were in the range of $10^5$–$10^6$ M·min $^{-1}$ with the highest value for VX ($k_a$ = 8.92 × $10^6$), the next highest for soman ($k_a$ = 3.22 × $10^6$) and the lowest for sarin ($k_a$ = 0.39 × $10^6$), respectively. The inhibition rate is the same *in vitro* and *in vivo*; when the real inhibition *in vivo* and *in vitro* is compared, it is possible to assess the concentration of the nerve agent present in the blood stream. When administered intramuscularly it is about 70% of the dose administered.

*Key words:* whole blood cholinesterases; rat; nerve agents; inhibition *in vitro*

## INTRODUCTION

The toxicodynamics of nerve agents and organophosphates (OP) are explained by acetylcholinesterase (AChE, EC 3.1.1.7) inhibition at the cholinergic synapses and the consequent accumulation of neuromediator acetylcholine followed by metabolic disbalance and other changes (Marrs 1996, Eyer 2003, Bajgar 2004, Rotenberg and Newmark 2004, Wiener and Hoffmann 2004, Geoghegan 2006). Exposure to OP is characterized by four basic reactions in the organism: resorption, transport, metabolism and toxic effect (Bajgar 2004).

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Before the toxic action, the OP is transported by the blood stream to the target sites (the central and peripheral nervous systems). The transport system can be considered as a base for the further action of the OP, and it is the first main system on which the OP have an effect. This reaction is very important because a part of the OP is bound and this part of the administered dose is thus excluded from further toxic action at the target sites, as is a further part that in some compounds is detoxified: thus, G agents (sarin, soman etc.) are, and V agents (VX, Russian VX etc.) are not detoxified in the organism (Bajgar 1991).

In the transport system (blood), two cholinesterases are present; AChE in the erythrocytes and butyrylcholinesterase (BuChE, EC 3.1.1.8) in the plasma. Both enzymes are inhibited during the transport of the OP according to the principle of "first come, first served" (Benschop and de Jong 2001), and therefore this inhibition can be considered as a good marker of poisoning with cholinesterase inhibitors (Bajgar et al. 2006). The partitioning between these two enzymes is influenced by the affinity of AChE and BuChE to the nerve agents. There are other

enzymes and proteins binding the nerve agents: carboxylesterases (inhibited by the agents) and blood proteins – particularly albumin – (binding the agents), with, however, significantly lower affinity than nerve agents (Bajgar 1991, 2004). The inhibition of both cholinesterases in blood depends mostly on:
– the affinity of OP to the cholinesterases in blood;
– the rate of penetration of the OP from the route of administration to the transport system;
– the detoxification of the OP.

The affinity of OP to cholinesterases can easily be tested *in vitro*, and this approach is frequently evaluated in the research of new reactivators. Although there are many studies on the inhibition and reactivation of cholinesterases *in vitro* (Kuča et al. 2004, 2005, Patočka et al. 2005, Musílek et al. 2007, Petroianu and Kalasz 2007, Hasan et al. 2008, Petroianu and Lorke 2008), there is a lack of complex data on the most usual and available animal – the rat – for all tissues of interest. There are separate data on the affinity *in vitro* of different OP to: human (Jandorf et al. 1955, Tammelin 1957, Aquilonius et al. 1964, Patočka and Tulach 1969, Aurbek et al. 2006); pig (Bajgar et al. 1971, Aurbek et al. 2006); erythrocyte AChE, and plasma BuChE (Tammelin 1957, Aquilonius et al. 1964, Back 1969, Bajgar et al. 1971), or brain AChE (Back 1969, Patočka and Bajgar 1971, Bajgar and Patočka 1977) etc. Inhibition constants inform us about the kinetics of the enzymes mentioned but do not correspond to the real status, i.e. to the whole inhibition capacity in the blood. However, this information is necessary for comparison of *in vivo* and *in vitro* results. The aim of this study is to obtain inhibition constants characterizing the affinity of the most common nerve agents – sarin, soman and VX – to the blood cholinesterases (plasma BuChE and erythrocyte AChE determined as the activity in the whole blood), *in vitro* using one method (one substrate, pH, conditions etc.) and *in vivo* (the rat).

## MATERIAL AND METHODS

*Animals*

6 female Wistar rats of the same litter (BioTest, Konárovice, Czech Republic), weighing 200–220 g, were used. The animals were housed in the Central Vivarium of the Faculty of Military Health Sciences under veterinary control. All the experiments were performed with the permission and under the supervision of the Ethics Committee of the Faculty of Military Health Sciences, Hradec Králové (permission No 153/06) according to § 17 of Czech law No. 207/2004, permission of responsible person 0001/94 – M 699. The animals were exsanguinated and the heparinized blood was used for the experiments.

*Chemicals*

Nerve agents were obtained from the Military Technical Institute of Protection (Brno, Czech Republic). They were of minimally 98% purity and stored in glass ampullas (1 ml). The solutions for experiments were prepared immediately before use.

*Determination of cholinesterase activity/inhibition*

Inhibition of the blood samples was performed using a solution of the relevant nerve agent (sarin, soman, VX) in isopropanol, a mixture of the whole blood (495 µl) and the inhibitor solution (5 µl). The final concentration of the inhibitor was $10^{-6}$, $10^{-7}$ a $10^{-8}$ M, and the final concentration of the solvent was 1% (v/v). At different time intervals (0, 3, 6, 10, 15, 20, 30 min), an aliquot part of the blood sample (50 µl) was haemolysed by adding of 950 µl distilled water. After 1 min, AChE activity was determined according to Ellman et al. (1961) as follows: 100 µl of the haemolysate was mixed with 1700 µl DTNB solution (1 mM solution of DTNB in 0.1 M TRIS-HCl buffer, pH 7.6) and an enzymatic reaction was started by adding 200 ul of substrate solution (1 mM acetylthiocholine in distilled water). The mixture was stabilized for 3 min and then the absorbancy was monitored at 436 nm/min (the delay is necessary to exclude false positive results caused by the titration of free SH-groups interfering with the activity determination).

*Statistical evaluation*

The dependence of cholinesterase activity changes vs. time was evaluated by the least square method in semi-logarithmical transformation, using relevant PC programmes: relative activity ($\Delta$ absorbancy$_{436\ nnm}$) was recalculated to per cent values (activity at the time zero represents 100%). Evaluation of the data was carried out using Microsoft Excel 2002 and GraphPad Prism version 4 for Windows, GraphPad Software, San Diego, California, USA (www.graphpad.com). For each determination (time interval, concentration of inhibitor), the mean from 3 measurements was used.

## RESULTS

Absolute values of cholinesterase activity in the rat blood varied from 10.1 to 15.5 µkat/l, with an average 11.3 ± 1.12 µkat/l (1 kat = $60 \cdot 10^6$ unit; unit/l = 16.67 nkat/l).

Changes of the activity in the blood after incubation with sarin are shown in Fig. 1. The inhibition is dependent on the inhibitor concentration and the time of incubation. Semilogarithmic transformation of the data allows a determination of the half-life of inhibition as shown in Fig. 2.

The bimolecular rate constants ($k_a$) were calculated according to the equation

$$\frac{0.69}{[I] \cdot t_{0.5}} = k_a \text{ (equation I);}$$

the same approach was used for calculation of $k_a$ values for soman and VX. They are summarized in Table 1. According to the bimolecular rate constants, VX seems to be the most potent inhibitor of rat blood cholinesterases followed by sarin and soman. From these data, it is possible to calculate the $IC_{50}$ constant, i.e. the inhibitor concentration causing 50% of inhibition. The values for 10 min inhibition are also shown in Table 1. It is clear that the order of $IC_{50}$ values is the same (sarin < soman < VX) but they are given for easier comparison with the data in the literature.

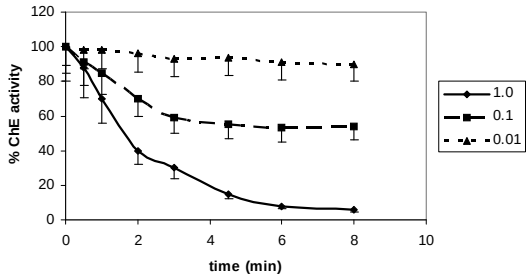


Fig. 1. **Inhibition of cholinesterases in the rat blood by different concentrations of sarin *in vitro*.** Sarin concentrations: 0.01–0.1 and 1.0 μM.

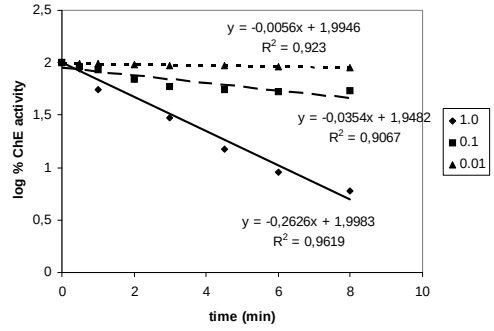


Fig. 2. **Inhibition of blood cholinesterases by different concentrations of sarin *in vitro* in semilogarithmic transformation.** Sarin concentrations: 0.01–0.1 and 1.0 μM.

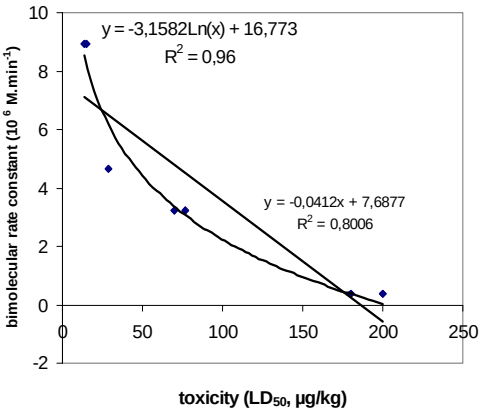


Fig. 3. **Correlation between toxicity expressed as $LD_{50}$ (i.m., μg/kg) and bimolecular rate constants for sarin, soman, VX** (our data), **and O-ethyl 2-(2-dimethylaminoethyl) methyl phosphonothiolate** (data of Bajgar 1991) **in semilogarithmical and linear transformation.** The correlation coefficients ($R^2$) are different for different transformations; higher $R^2$ for semilogarithmic transformation indicates that this dependence will be probably more significant.

## DISCUSSION

The inhibition of erythrocyte AChE of different species has been described many times, and the eryhtrocyte source has mostly been human, pig or rat; e.g. Aurbek et al. (2006) determined the bimolecular rate constant ($k_a$) for human erythrocyte AChE to be $9.91 \cdot 10^7$ and for pig erythrocyte AChE and VX, $4.43 \cdot 10^7$ M · min$^{-1}$. These constants are higher than those obtained in our experiments: this could be caused by the use of a different enzyme source and different contents of AChE and BuChE in the blood. This ratio for rat blood (7:3) was determined in an earlier study (71% for erythrocyte AChE and 29% for plasma BuChE) (Bajgar 1972). The sensitivity of blood cholinesterases to sarin and VX has been described – for human erythrocyte AChE – as about 10 times higher for VX in comparison with sarin (Worek et al. 2004). Jandorf et al. (1955) determined the bimolecular rate constant for rat erythrocyte AChE to be approximately $10^7$, and this is in agreement with our data. The inhibition constant occurring in the literature more frequently – $IC_{50}$ – can be derived from the equation (I), but this constant is very dependent on the time of incubation of the enzyme with an inhibitor. Among other tissues studied for AChE inhibition, the data for human

Table 1. **Inhibition constants for interaction of the rat blood cholinesterases with nerve agents.**

| Constant | Sarin | Soman | VX |
|---|---|---|---|
| $k_a \cdot 10^6$ ($M \cdot min^{-1}$) | 0.39 ± 0.26 | 3.22 ± 0.88 | 8.92 ± 1.03 |
| $I_{50} \cdot 10^{-7}$ (M) | 176 | 22.4 | 7.7 |
| LD (μg/kg, i.m.)* | 200 (180) | 70 (77) | 13.6 (15) |

* Toxicity data according to Bajgar (2004) and the reference Vachek et al. (1996).

erythrocyte AChE activity are as follows: Back (1969) determined $IC_{50}$ for human plasma and soman $10^{-7}$–$10^{-8}$ M, whereas the same constant for pig erythrocyte AChE was $10^{-9}$ M (Bajgar et al. 1971) and for human plasma BuChE $10^{-8}$ M (Tammelin 1957, Aquilonius et al. 1964, Patočka and Tulach 1969, Bajgar and Patočka 1977). The correlation between toxicity and bimolecular rate constants for sarin, soman, VX (our data), and O-ethyl 2-(2-dimethylaminoethyl) methyl phosphonothiolate (Bajgar 1992) is demonstrated in Fig. 3.

If we compare the inhibition potency characterized by $k_a$ constant with the toxicity expressed as $LD_{50}$ (Vachek et al. 1996, Bajgar et al. 2007), a good correlation can be found (Fig. 3). In all these comparisons it is necessary to point out that inhibition of cholinesterases in the blood is an indicator of exposure but it is not connected with the mechanism of the action of nerve agents: it has been demonstrated that inhibition of erythrocyte AChE in rabbits does not influence normal functions of the organism (Kassa et al. 1997, Kassa and Bajgar 1999). However, the inhibition in the transport system can be very good marker of not only exposure level but also a marker for assessment of nerve agent concentration in the blood stream. This was our purpose in making further calculations:

It was demonstrated that following administration of VX (i.m., $1 \cdot LD_{50}$, 13.6 μg/kg, i.e. $5.087 \cdot 10^{-8}$ mol/kg), the half life ($t_{0.5}$) of blood cholinesterase inhibition was determined to be 2.4 min (Bajgar et al. 2007). When this constant is introduced into the equation (I), the concentration of inhibitor (VX) was calculated as $3.667 \cdot 10^{-8}$ mol/l/kg, and it is 72.1% of the dose administered. This means that after i.m. administration, about 70% of the dose administered is present in the blood stream. Using this approach, it would be possible to calculate a dose of inhibitor present in the blood after intoxication with different routes of administration, and thus, to improve our knowledge of the mechanism of the action of these highly toxic agents.

## CONCLUSIONS

For studies dealing with the effect of nerve agents, whole blood is the material of choice for inhibition studies.

For kinetic studies, the use of separated enzymes is recommended.

The inhibition of cholinesterases in whole blood allows an assessment of the concentration of inhibitor in the blood stream.

Toxicity correlates well with the rate of cholinesterase inhibition in the blood.

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