

Original article

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Translation of *in vitro* to *in vivo* pyridinium oxime potential in tabun poisoning

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Even if organophosphorus (OP) nerve agents were banned entirely, their presence would remain a problem as weapons of terror (like in Syria). Oxime antidotes currently used in medical practice still fall short of their therapeutic purpose, as they fail to fully restore the activity of cholinesterases, the main target for OPs. As orphan drugs, these antidotes are tested too seldom for anybody's benefit. Over the last few decades, search for improved reactivators has reached new levels, but the translation of data obtained *in vitro* to *in vivo* application is still a problem that hinders efficient therapy. In this study, we tested the strengths and weaknesses of extrapolating pyridinium oxime antidotes reactivation efficiency from *in vitro* to *in vivo* application. Our results show that this extrapolation is possible with well-determined kinetic constants, but that it also largely depends on oxime circulation time and its tissue-specific distribution. This suggests that pharmacokinetic studies should be planned at the early stages of antidote development. Special attention should also be given to improving oxime distribution throughout the organism to overcome this major constraint in improving overall OP therapy.

KEY WORDS: *acetylcholinesterase; antidotes; butyrylcholinesterase; K048; nerve agents; pharmacokinetics, tissue-specific activity*

Exposure to organophosphorus compounds (OP) such as nerve agents and certain pesticides causes diverse health complications including nervous, endocrine, reproductive, and immune system changes (1, 2). Their acute toxicity is mainly the result of the inhibition of acetylcholinesterase (AChE; EC 3.1.1.7) activity in the central and peripheral nervous system. Current treatment relies on the use of anticholinergic drug atropine to reduce the effects of accumulating acetylcholine usually combined with oximes that reactivate phosphorylated AChE (3-5). However, the potential of the oximes used in medical practice today, such as 2-PAM, HI-6, and obidoxime, is limited primarily due to the very specific properties of each of the enzyme-OP conjugates (6-9).

Just as any other drug, oximes have to comply with a great number of requirements to be accepted for human use. Antidotes of this kind fall into the category of orphan drugs, which means that trials are seldom performed. Even preclinical studies on animal models are limited due to ethical issues and high security regulations for work with OPs (1). Therefore, selecting the right compound for further studies based on promising *in vitro* results undoubtedly presents the greatest challenge. This is even more true when we consider that so many results available today still lack a critical angle on the oximes' antidotal potencies and how

applicable they are *in vivo*. This kind of information would greatly help to develop more effective therapies against OP poisoning.

With this in mind, we tested the strengths and weaknesses of extrapolating pyridinium oxime antidote results from *in vitro* to *in vivo* application. For our study, we chose the oxime K048 and OP compound tabun, since previous antidotal research on tabun highlighted oxime K048 as the leading pyridinium reactivator of tabun-inhibited AChE (10-13). As there is strong *in vitro* and *in vivo* evidence favouring this oxime-OP model, we believed that it would serve well for translation of the oximes' potential as reactivator of OP-inhibited AChE to its antidotal potential against OP poisoning.

We focused on oxime distribution, elimination, and tissue-specific AChE reactivation capacity in rats exposed subcutaneously to a single sub-lethal dose of tabun (3/4 LD₅₀). This route and dose were chosen to ensure toxic manifestations at all levels without loading the animals with excess poison (that would mask the effect of the oxime). Oxime K048 was applied intraperitoneally in a single dose approximating the *in vitro* determined enzyme-oxime (K_i) or phosphorylated enzyme-oxime (K_{ox}) dissociation constant (10, 12), calculated on the estimated whole body fluid content (15). This dose ensures oxime concentration that efficiently reactivates AChE *in vitro* (10).

The tissue in which we monitored reactivation determines the final outcome of OP poisoning: blood,

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diaphragm, and brain. To be more precise, cholinesterase activity in blood is used as a marker of exposure to OPs and is considered to be the first line of defence before OPs reach vital sites (5, 16, 17). AChE inhibition in diaphragm leads to life-threatening difficulties in breathing, whereas inhibition in the brain has severe and long-term consequences, all of which lead to death.

We also followed the activity of butyrylcholinesterase (BChE, EC 3.1.1.8), a stoichiometric scavenger of OPs (5, 16, 18, 19). Much effort has been put so far into improving BChE's endogenous scavenging capacity, mainly by trying to convert it into a pseudo-catalytic scavenger by adding a BChE reactivation-specific oxime. Certain progress has been made with K048 analogues, pyridinium oximes K127 and K117, against tabun (20) and with some non-pyridinium oximes against VX-, cyclosarin-, and paraoxon-BChE conjugates (21). Although our oxime model should be specific to AChE reactivation (11), data obtained on BChE will give additional information for the extrapolation of *in vitro* pyridinium oxime efficiency to its efficiency *in vivo*.

METHODS

Chemicals

Oxime K048 (1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium) butane dibromide) ($M_r=460.16 \text{ g mol}^{-1}$) was a gift from Dr Kamil Kuća, University of Hradec Kralove, Czech Republic. It was kept at room temperature and dissolved in water or atropine immediately before use. Tabun (ethyl *N,N*-dimethyl phosphoramidocyanidate) was purchased from NC Laboratory, Spiez, Switzerland. A stock solution of $5,000 \mu\text{g mL}^{-1}$ was prepared in isopropyl alcohol. Further dilutions were made in saline immediately before use. Atropine sulphate (Kemika, Zagreb, Croatia) was dissolved in water. Non-ionic detergent 1 % Triton X-100 was purchased from SERVA Electrophoresis GmbH, Heidelberg, Germany. Acetylthiocholine iodide (ATCh), DTNB (5,5'-dithiobis(2-nitrobenzoic acid)), BW284C51 (1,5-bis(4-allyldimethyl-ammoniumphenyl)-pentane-3-one dibromide), ethopropazine ((10-[2-diethylaminopropyl] phenothiazine)hydrochloride), and chemicals for high performance liquid chromatography (HPLC) [acetonitrile gradient grade LiChrosolv, disodium hydrogen phosphate dihydrate, citric acid monohydrate, ethylenedinitrilo tetraacetic acid disodium salt dihydrate (Na_2EDTA), 1-octane sulphonic acid sodium salt, perchloric acid (70 %), and phosphoric acid] were purchased from Sigma-Aldrich (St. Louis, MO, USA). The water used for HPLC was double-distilled and deionised HPLC-grade.

Animal treatment and tissue isolation

Adult male Wistar rats (240-280 g body weight) were kept in Macrolone cages under controlled conditions (room

temperature $21 \text{ }^\circ\text{C}$, light and dark cycles exchanging every 12 h), received a standard diet, and had free access to water. This study was performed with the approval of the Ethics Committee of the Institute for Medical Research and Occupational Health, Zagreb, Croatia and the Faculty of Military Health Sciences in Hradec Kralove, Czech Republic.

The rats were divided into five groups (four animals in each) for every time point (0.5 h, 1 h, and 6 h): (a) saline (control group); (b) K048; (c) tabun; (d) tabun + atropine; (e) tabun + atropine + K048. The first two groups (saline and K048 group) received intraperitoneally (i.p.) 2 mL kg^{-1} of saline or a $1/4 \text{ LD}_{50}$ dose of K048 ($\text{LD}_{50}=238.3 \text{ mg kg}^{-1}$ body weight; 22), respectively. A sub-lethal dose ($3/4 \text{ LD}_{50}$) of tabun ($\text{LD}_{50}=317.5 \mu\text{g kg}^{-1}$ body weight; 22) was administered subcutaneously (s.c.) to the three groups receiving tabun. One minute after tabun exposure, the tabun + atropine group received a 10 mg kg^{-1} body weight i.p. dose of atropine, while the tabun + atropine + K048 group received a combination of $1/4 \text{ LD}_{50}$ K048 and atropine (10 mg kg^{-1}).

At time points 0.5 h, 1 h, and 6 h, blood samples were taken directly from the heart. Plasma was separated by centrifugation and stored at $-20 \text{ }^\circ\text{C}$ until analysis. After isolation, the brain and diaphragm were rinsed with saline and stored at $-20 \text{ }^\circ\text{C}$ until analysis. After thawing, homogenates were prepared as 100 mg mL^{-1} diaphragm homogenate and 40 mg mL^{-1} brain homogenate, diluted in a corresponding volume of 0.1 mol L^{-1} sodium phosphate buffer, pH 7.4 (supplemented with 1 % Triton X-100 for the diaphragm sample preparation). The tissue homogenates were prepared using an IKA-Werke ultra turrax homogeniser (LABSCO, Friedberg, Germany). The homogenates were centrifuged at 3000 g and $4 \text{ }^\circ\text{C}$ for 10 min. The supernatants were used for the cholinesterase enzyme activity measurement promptly after the preparation (23).

Pharmacokinetics

The pharmacokinetics of K048 was determined in a group of six male Wistar rats for each time point. This group received the same dose as the K048 group for cholinesterase activity measurement ($1/4 \text{ LD}_{50}$; i.p.). The animals were narcotised with carbon dioxide, and blood taken from the heart through the thoracic cavity into heparin-coated tubes, gently mixed, and centrifuged at 3000 g and $10 \text{ }^\circ\text{C}$ for 15 min (Universal 320R, Hettich, Germany) to obtain plasma. The animals were perfused transcardially with saline (0.9 % NaCl) for 8 min (50 mL min^{-1}). After perfusion, the tissues (heart, diaphragm, kidney, hind-limb muscle, and brain) were carefully removed. All of the samples were stored at $-80 \text{ }^\circ\text{C}$ until HPLC analysis. The proteins were directly precipitated from plasma samples with perchloroacetic acid (300:25). The tissues were homogenised (using Ultra Turrax, IKA-Werke for 30 s and by UP50H, Hielsche Ultrasound Technology, Germany for

1 min) in purified water (1:4), and the proteins precipitated with perchloroacetic acid (300:25). The samples were centrifuged at 11,000 g and 10 °C for 10 min. The obtained supernatants were used for analyses.

HPLC separation conditions for K048 in plasma and tissue samples

Analytical column LiChrospher® 60 RP-select B (5 µm) LiChroCART® 125-4 (Merck, Darmstadt, Germany) with guard column LiChrospher® 60 RP-select B (5 µm) was used for analysis. The mobile phase contained 56.2 mmol L⁻¹ Na₂HPO₄, 47.9 mmol L⁻¹ citric acid, 0.027 mmol L⁻¹ Na₂EDTA, 0.925 mmol L⁻¹ octane sulphonic acid, and 75:950 acetonitrile:aqueous phase. The flow rate of the mobile phase was 1 mL min⁻¹. The pH was adjusted to 3.5 with H₃PO₄. Sample volume was 20 µL. All chromatograms were obtained at 40 °C. Samples were analysed with UV detection. The maximum wavelength of K048 was 275 nm.

All pharmacokinetic analyses were performed on an Agilent 1260 Series liquid chromatograph (Palo Alto, CA, USA) composed of degasser, quaternary pump, light-tight autosampler unit set, thermostated column compartment, and MS detector LCQ Fleet (Thermo Finnigan, San Jose, CA, USA). Agilent ChemStation software and statistical software Prism4 (GraphPad Software, San Diego, CA, USA) were used for analysis of results.

Enzyme activity assay

Plasma, diaphragm, and brain samples were analysed for AChE and BChE activity using the spectrophotometric Ellman method (24, 25). Final sample dilutions for the assay were: plasma - 50 times; brain - 625 times; and diaphragm - 66.7 times. AChE and BChE activities were distinguished using AChE- and BChE-selective inhibitors BW284C51

(10 µmol L⁻¹) and ethopropazine (20 µmol L⁻¹), respectively (26, 27). The activity was measured in a 0.1 mol L⁻¹ sodium phosphate buffer, pH 7.4, at 25 °C using ATCh (1.0 mmol L⁻¹) and DTNB (0.3 mmol L⁻¹). All of the measurements were performed on a Cary 300 spectrophotometer (Varian Inc, Australia). For diaphragm samples increase in absorbance was monitored at 436 nm and for plasma and brain samples at 412 nm over 4 min. Enzyme activity was expressed as micromoles of ATCh hydrolysed per minute and millilitre of tissue homogenate (undiluted) or plasma sample from which ratio to control activity was calculated (% of control activity). For the statistical analysis we used one-way analysis of variance with Bonferroni correction (*post hoc*). Values *p*<0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Figure 1 summarises K048 distribution and elimination across tissues. As a positively charged compound, K048 is quickly eliminated from the organism (28-30). In our study the entire dose (60 mg kg⁻¹ body weight) was eliminated within a few hours from i.p. application. This could be seen as an advantage of pyridinium oxime-based antidotes, but at the same time as a disadvantage considering effective therapy and dosage regimes. Moreover, earlier studies by Thiermann et al. (17, 31) have argued in favour of repeated or continuous infusion with a pyridinium oxime antidote to counter the persistence of OPs in the organism and ensure full recovery and survival.

K048's distribution profile shows that it reaches the highest concentration of ≈200 µmol L⁻¹ in the plasma 30 min after i.p. injection (Table 1). Considering AChE reactivation efficiency *in vitro* and the fact that pyridinium oximes are not metabolised in serum (32), this concentration

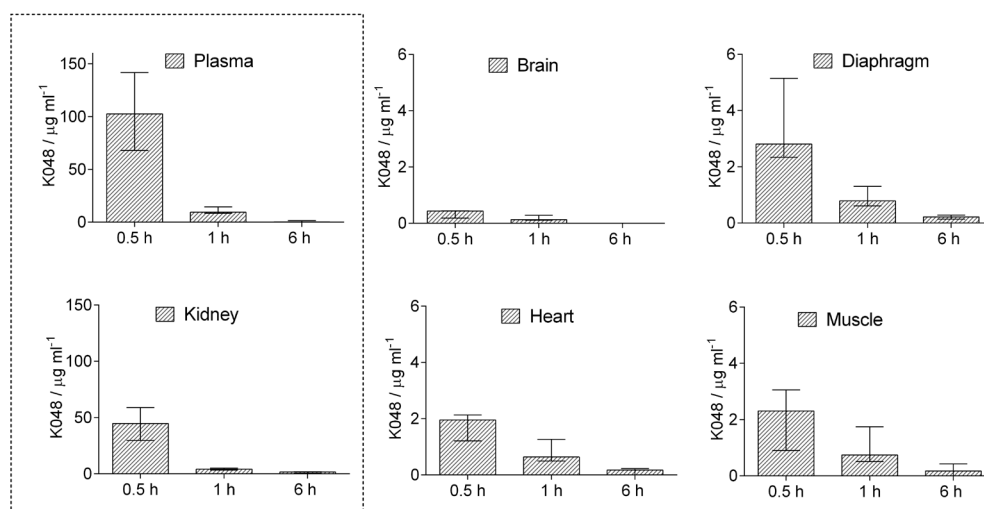


Figure 1 Rat plasma and tissue concentrations of K048 after a single i.p. dose of 238.3 mg kg⁻¹; (1/4 of the LD₅₀, see ref. 20). Data are presented as medians with interquartile ranges. Plasma and kidney are highlighted as tissues with the highest oxime concentrations

Table 1 Estimated AChE reactivation in the tissue calculated from the HPLC-determined maximal oxime K048 concentration (Figure 1) and based on constants obtained for K048-assisted reactivation *in vitro* and the equations describing reactivation

Tissue	Mean oxime concentration (determined in 30 min)		Recalculated k_{obs} (min^{-1})	Estimated AChE reactivation in 30 min
Plasma	102.6 $\mu\text{g mL}^{-1}$	223 $\mu\text{mol L}^{-1}$	0.0496	78 %
Brain	0.43 $\mu\text{g mL}^{-1}$	0.9 $\mu\text{mol L}^{-1}$	0.0006	2 %
Diaphragm	2.81 $\mu\text{g mL}^{-1}$	6.1 $\mu\text{mol L}^{-1}$	0.0039	11 %
Kidney	44.6 $\mu\text{g mL}^{-1}$	97 $\mu\text{mol L}^{-1}$	0.0347	64 %
Heart	1.95 $\mu\text{g mL}^{-1}$	4.2 $\mu\text{mol L}^{-1}$	0.0027	8 %
Muscle	2.31 $\mu\text{g mL}^{-1}$	5.0 $\mu\text{mol L}^{-1}$	0.0032	9 %

*Calculations were done using kinetic constants for hAChE: $k_{-2}=0.074 \text{ min}^{-1}$, $K_{OX}=110 \mu\text{mol L}^{-1}$, and equations: $\ln([EP]_t/[EP]_0)=k_{obs} \cdot t$ and $k_{obs}=k_{-2} \cdot [OX]/(K_{OX}+[OX])$; where $[EP]_0$ and $[EP]_t$ are the concentrations of phosphorylated enzyme at time zero and at time t , respectively; k_{obs} is the observed first-order rate constant of reactivation at any given oxime concentration $[OX]$, K_{OX} is the dissociation constant of the phosphorylated enzyme-oxime complex and k_{-2} is the maximum first-order reactivation rate constant (10)

of K048 should suffice for AChE reactivation *in vivo* (10). The second highest concentration of K048 was observed in the kidneys ($\approx 100 \mu\text{mol L}^{-1}$), also half an hour after i.p. injection. This is not unusual, since renal clearance is the main elimination pathway of pyridinium oximes (28, 32). If we look at the other organs such as the diaphragm, skeletal muscle, and heart, we can see that positively charged pyridinium compounds have difficulty crossing biological barriers and not just the blood-brain barrier (maximum brain concentration was less than $1 \mu\text{mol L}^{-1}$; 33). The problem of delivering oxime antidotes to the brain has been addressed by many approaches so far. Several groups of researchers have tried to synthesise uncharged oximes that would be able to cross the blood-brain barrier (34-38), but these new classes of oximes fall short of pyridinium oxime efficiency when it comes to tabun reactivation (37, 39). Much research has also been done to improve or modify

transport of oximes across the blood-brain barrier. Some used membrane transporters by linking an oxime to a sugar moiety and some employed nanoparticles to act as carriers (40, 41). Resolving distribution of an antidote across the organism perhaps requires different approach, not necessarily limited to modifying oxime structure.

To get an idea of what we can expect in the sense of *in vivo* reactivation efficiency, we made calculations (Table 1) based on reactivation constants obtained *in vitro* (10). These calculations indicate that with the obtained concentrations an efficient AChE reactivation of 80 % in the first 30 min could be expected only in the plasma/blood of tabun poisoned rats.

Table 2 shows cholinesterase activity *in vivo* in the control groups (animals that received saline only), which serve as the baseline. Tissue-specific reactivation of AChE and BChE activity in treated animals over time is given in

Table 2 AChE and BChE activities in control rats ($n=4$)

Enzyme	Tissue	Time (h)	Activity range ($\mu\text{mol mL}^{-1} \text{ min}^{-1}$)	Average activity \pm SD ($\mu\text{mol mL}^{-1} \text{ min}^{-1}$)
AChE	plasma	0.5	0.078-0.179	0.117 \pm 0.044
		1	0.084-0.204	0.122 \pm 0.056
		6	0.110-0.209	0.137 \pm 0.048
	brain	0.5	6.540-7.338	7.046 \pm 0.376
		1	6.390-8.259	7.396 \pm 0.776
		6	5.044-6.425	5.779 \pm 0.566
	diaphragm	0.5	0.446-0.887	0.646 \pm 0.182
		1	0.321-0.739	0.580 \pm 0.182
		6	0.470-0.766	0.633 \pm 0.125
BChE	plasma	0.5	0.064-0.095	0.079 \pm 0.014
		1	0.041-0.105	0.063 \pm 0.029
		6	0.045-0.090	0.072 \pm 0.020
	diaphragm	0.5	0.148-0.204	0.166 \pm 0.026
		1	0.129-0.244	0.185 \pm 0.048
		6	0.139-0.292	0.224 \pm 0.068

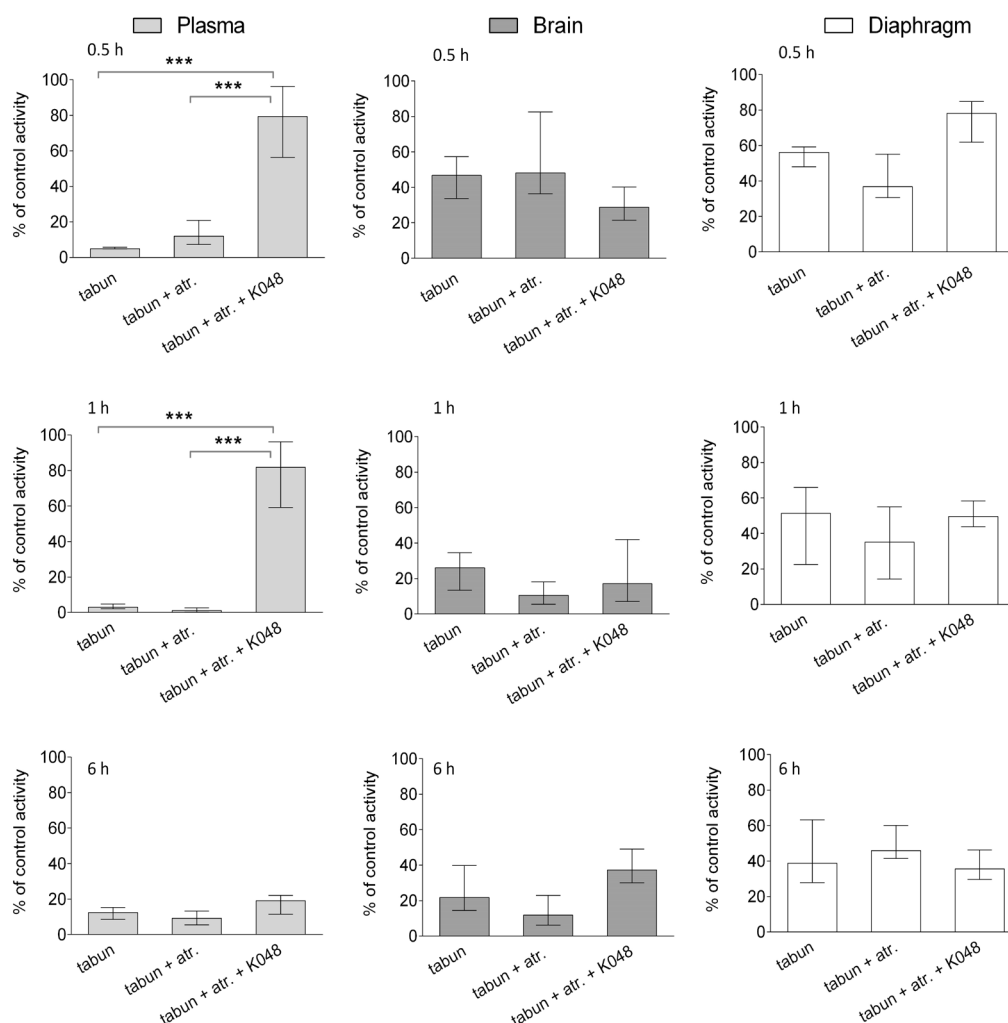


Figure 2 The effects of oxime K048 on AChE activity in the plasma, brain, and diaphragm of tabun-poisoned rats after 0.5, 1, and 6 h of therapy. Activity is expressed as the percentage of mean control activity (Table 2). K048 alone did not affect AChE activity and is not shown. Data are presented as medians with interquartile ranges. Statistical significance was determined using one-way analysis of variance and Bonferroni correction. Differences were considered significant when $p < 0.05$

Figures 2 and 3, respectively, and is expressed as percentage of the baseline. In rat plasma, AChE activity contributed to the total measured activity with 60 %, while the remaining 40 % was assigned to BChE. This ratio was probably due to the presence of free AChE in the plasma and partial haemolysis of rat erythrocytes before centrifugation of the whole blood. In the diaphragm, AChE activity accounted for almost 80 % of the total measured activity, while in the rat brain, AChE accounted for 95 % of the total measured activity, which is why we omitted BChE from evaluation in the brain.

Therapy with K048 was effective in restoring AChE activity only in plasma; the highest reactivation of around 80 % was reached within the first 30 min (Figure 2). With time and elimination (cf. Figure 1) this effect diminished. At 6 h, AChE activity dropped to the level measured in rats treated with tabun alone. In other words, the remaining tabun in plasma continued to inhibit the reactivated AChE.

This finding confirms that it is vital to monitor AChE plasma activity during therapy and to adapt and repeat antidote dosing not only to keep AChE activity recovered but also to bioscavenge the remaining OP with the antidote before it affects the brain and other vital organs. In the diaphragm or the brain K048 did not significantly restore enzyme activity. These results confirm our extrapolation of the *in vitro* to *in vivo* data based on the oxime's tissue-specific concentrations.

In addition, it was interesting to see the extent of AChE inhibition in each tissue (cf. Fig. 2). In plasma it was significant. In the brain, AChE inhibition was evident shortly after tabun injection, but it did not drop below 30 % of the control group. In the diaphragm, AChE inhibition was not as severe as would be expected and stayed within 40-50 % of the control value at all time points. A similar tissue-specific distribution was reported for other nerve agents like sarin, cyclosarin, VR, and VX (42).

According to *in vitro* data, K048-assisted reactivation of tabun-inhibited BChE should be insignificant (11). In other words, kinetic constants of $k_{\max} = 0.0022 \text{ min}^{-1}$ and $K_{\text{OX}} = 630 \mu\text{mol L}^{-1}$ predict a reactivation maximum of only 2 % with pharmacokinetically determined oxime plasma concentration ($230 \mu\text{mol L}^{-1}$ in 30 min, cf. Table 1). Indeed, according to the *in vivo* results (Figure 3), K048 therapy did not preserve BChE activity in any of the tested samples, which confirms that BChE in combination with K048 can act only as a stoichiometric scavenger of tabun and not as a catalytic one.

In conclusion, our results show that it is possible to correlate *in vitro* to *in vivo* AChE or BChE reactivation efficiency based only on well-determined kinetic constants. This approach should be applicable for future research of pyridinium oximes. However, this extrapolation would not be possible without us knowing the exact concentration of the oxime in a specific tissue after application. Therefore, pharmacokinetic studies should be planned for the early stages of antidote development. Pharmacokinetic profile can also be predicted with *in silico* models as a way to select a promising compound before performing *in vivo* experiments (43).

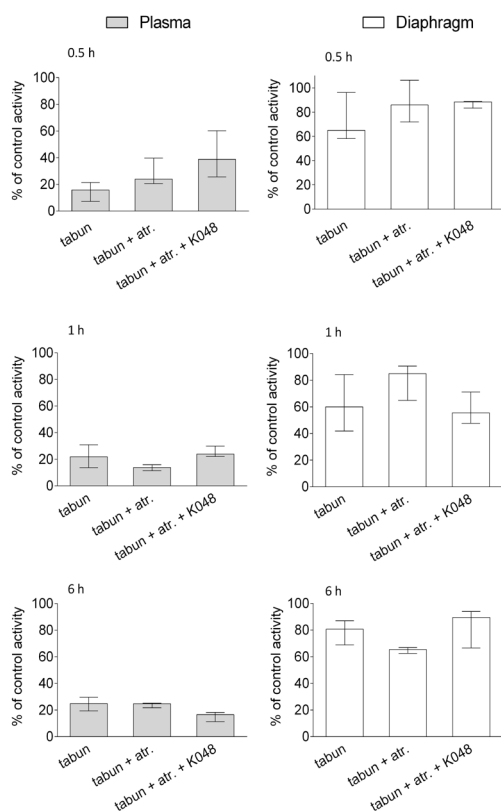


Figure 3 The effects of oxime K048 on BChE activity in the plasma and diaphragm of tabun-poisoned rats after 0.5, 1, and 6 h of therapy. Activity is expressed as the percentage of mean control activity (Table 2). K048 alone did not affect BChE activity and is not shown. Data are presented as medians with interquartile ranges. Statistical significance was determined using one-way analysis of variance and Bonferroni correction. Differences were considered significant when $p < 0.05$

Other possible *in vitro* effects beyond cholinesterase reactivation are also concentration-dependant (44, 45), such as muscle regeneration efficiency, interaction with cholinergic receptors, cell viability, and oxidative stress, all of which can contribute to or diminish the outcome of therapy. Furthermore, future oxime development should focus on improving distribution across the organism to overcome maybe the main hindrance in improving therapy. Current antidotal research mostly revolves around passive diffusion linked to lipophilicity (46, 47). This perspective requires changing the oxime's structure to obtain more desirable properties, but such changes greatly affect the oxime's potency to reactivate inhibited cholinesterases. A step in the right direction could be to consider active transport or involvement of transporters as a way of improving therapy (48). Maybe in doing so, and based on wide experimental background obtained so far, we can reach a milestone we set out to achieve decades ago.

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REFERENCES

- Gupta R. Toxicology of Organophosphate and Carbamate Compounds. 1st ed. London: Elsevier Academic Press; 2005.
- Malekirad AA, Faghieh M, Mirabdollahi M, Kiani M, Fathi A, Abdollahi M. Neurocognitive, mental health, and glucose disorders in farmers exposed to organophosphorus pesticides. *Arh Hig Rada Toksikol* 2013;64:1-7. doi: 10.2478/10004-1254-64-2013-2296
- Gray AP. Design and structure-activity relationships of antidote to organophosphorus anticholinesterase agents. *Drug Metab Rev* 1984;15:557-89. doi: 10.3109/03602538409029973
- Dawson MR. Review of oximes available for treatment of nerve agent poisoning. *J Appl Toxicol* 1994;14:317-31. PMID: 7822680
- Doctor BP, Maxwell DM, Ashani Y, Saxena A, Gordon RK. New approaches to medical protection against chemical warfare nerve agents. In: Somani SM, Romano JA Jr, editors. Chemical warfare agents: toxicity at low levels. New York (NY): CRC Press; 2002. p. 191-214.
- Kovarik Z, Radić Z, Berman HA, Simeon-Rudolf V, Reiner E, Taylor P. Mutant cholinesterases possessing enhanced capacity for reactivation of their phosphorylated conjugates. *Biochemistry* 2004;43:3222-9. PMID: 15023072

7. Masson P. Evolution of and perspectives on therapeutic approaches to nerve agent poisoning. *Toxicol Lett* 2011;206:5-13. doi: 10.1016/j.toxlet.2011.04.006
8. Wilhelm CM, Snider TH, Babin MC, Jett DA, Platoff GE Jr, Yeung DT. A comprehensive evaluation of the efficacy of leading oxime therapies in guinea pigs exposed to organophosphorus chemical warfare agents or pesticides. *Toxicol Appl Pharmacol* 2014;281:254-65. doi: 10.1016/j.taap.2014.10.009
9. Kovarik Z, Maček Hrvat N, Katalinić M, Sit RK, Paradyse A, Žunec S, Musilek K, Fokin VV, Taylor P, Radić Z. Catalytic soman scavenging by the Y337A/F338A acetylcholinesterase mutant assisted with novel site-directed aldoximes. *Chem Res Toxicol* 2015;28:1036-44. doi: 10.1021/acs.chemrestox.5b00060.
10. Čalić M, Lucić Vrdoljak A, Radić B, Jelić D, Jun D, Kuća K, Kovarik Z. *In vitro* and *in vivo* evaluation of pyridinium oximes: Mode of interaction with acetylcholinesterase, effect on tabun- and soman poisoned mice and their cytotoxicity. *Toxicology* 2006;219:85-96. doi: 10.1016/j.tox.2005.11.003
11. Lucić Vrdoljak A, Čalić M, Radić B, Berend S, Kuća K, Kovarik Z. Pretreatment with pyridinium oximes improves antidotal therapy against tabun poisoning. *Toxicology* 2006;228:41-50. doi: 10.1016/j.tox.2006.08.012
12. Kovarik Z, Čalić M, Šinko G, Bosak A. Structure-activity approach in the reactivation of tabun-phosphorylated human acetylcholinesterase with bispyridinium *para*-oximes. *Arh Hig Rada Toksikol* 2007;58:201-9. doi: 10.2478/v10004-007-0013-7
13. Kovarik Z, Čalić M, Bosak A, Šinko G, Jelić D. *In vitro* evaluation of aldoxime interactions with human acetylcholinesterase. *Croat Chem Acta* 2008;81:47-57.
14. Katalinić M, Kovarik Z. Reactivation of tabun-inhibited acetylcholinesterase investigated by two oximes and mutagenesis. *Croat Chem Acta* 2012;85:209-12. doi: 10.5562/cca1815
15. Foy JM, Schnieden H. Estimation of total body water (virtual tritium space) in the rat, cat, rabbit, guinea-pig and man, and of the biological half-life of tritium in man. *J Physiol* 1960;154:169-76. doi: 10.1113/jphysiol.1960.sp006571
16. Saxena A, Sun W, Luo C, Myers TM, Koplovitz I, Lenz DE, Doctor BP. Bioscavenger for protection from toxicity of organophosphorus compounds. *J Mol Neurosci* 2006;30:145-7. doi: 10.1385/JMN:30:1:145
17. Thiermann H, Eyer F, Felgenhauer N, Pfab R, Zilker T, Eyer P, Worek F. Pharmacokinetics of obidoxime in patients poisoned with organophosphorus compounds. *Toxicol Lett* 2010;197:236-42. doi: 10.1016/j.toxlet.2010.06.005
18. Lenz DE, Maxwell DM, Koplovitz I, Clark CR, Capacio BR, Cerasoli DM, Federko JM, Luo C, Saxena A, Doctor BP, Olson C. Protection against soman or VX poisoning by human butyrylcholinesterase in guinea pigs and cynomolgus monkeys. *Chem Biol Interact* 2005;157-158:205-10. PMID: 16289064
19. Rosenberg YJ, Laube B, Mao L, Jiang X, Hernandez-Abanto S, Lee KD, Adams R. Pulmonary delivery of an aerosolized recombinant human butyrylcholinesterase pretreatment protects against aerosolized paraoxon in macaques. *Chem Biol Interact* 2013;203:167-71. doi: 10.1016/j.cbi.2012.11.004
20. Kovarik Z, Katalinić M, Šinko G, Binder J, Holas O, Jung Y-S, Musilova L, Jun D, Kuća K. Pseudo-catalytic scavenging: searching for a suitable reactivator of phosphorylated butyrylcholinesterase. *Chem Biol Interact* 2010;187:167-71. doi: 10.1016/j.cbi.2010.02.023
21. Radić Z, Dale T, Kovarik Z, Berend S, Garcia E, Zhang L, Amitai G, Green C, Radić B, Duggan BM, Ajami D, Rebeck JJr, Taylor P. Catalytic detoxification of nerve agent and pesticide organophosphates by butyrylcholinesterase assisted with nonpyridinium oximes. *Biochem J* 2013;450:231-42. doi: 10.1042/BJ20121612.
22. Kovarik Z, Lucić Vrdoljak A, Berend S, Čalić M, Kuća K, Musilek K, Radić B. Evaluation of oxime K203 as antidote in tabun poisoning. *Arh Hig Rada Toksikol* 2009;60:19-26. doi: 10.2478/10004-1254-60-2009-1890
23. Camp S, Zhang L, Krejci E, Dobbertin A, Bernard V, Girard E, Duysen EG, Lockridge O, De Jaco A, Taylor P. Contributions of selective knockout studies to understanding cholinesterase disposition and function. *Chem Biol Interact* 2010;187:72-7. doi: 10.1016/j.cbi.2010.02.008
24. Ellman GL, Courtney KD, Andres V Jr, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 1961;7:88-90. doi: 10.1016/0006-2952(61)90145-9
25. Eyer P, Worek F, Kiderlen D, Sinko G, Stuglin A, Simeon-Rudolf V, Reiner E. Molar absorption coefficients for the reduced Ellman reagent: reassessment. *Anal Biochem* 2003;312:224-7. doi: 10.1016/S0003-2697(02)00506-7
26. Reiner E, Šinko G, Škrinjarčić-Špoljar M, Simeon-Rudolf V. Comparison of protocols for measuring activities of human blood cholinesterases by the Ellman method. *Arh Hig Rada Toksikol* 2000;51:13-8. PMID: 11059068
27. Simeon-Rudolf V, Šinko G, Štuglin A, Reiner E. Inhibition of human blood acetylcholinesterase and butyrylcholinesterase by ethopropazine. *Croat Chem Acta* 2001;74:173-82.
28. Kušić R, Bošković B, Vojvodić V, Jovanović D. HI-6 in man: blood levels, urinary excretion, and tolerance after intramuscular administration of the oxime to healthy volunteers. *Fundam Appl Toxicol* 1985;5(6part2):S89-S97. doi: 10.1093/toxsci/5.6part2.89
29. Kalász H, Hasan MY, Sheen R, Kuća K, Petroianu G, Ludányi K, Gergely A, Tekes K. HPLC analysis of K-48 concentration in plasma. *Anal Bioanal Chem* 2006;385:1062-7. PMID: 16763789
30. Zdarova Karasova J, Chladek J, Hroch M, Josef F, Hnidkova D, Kuća K. Pharmacokinetic study of two acetylcholinesterase reactivators, trimedoxime and newly synthesized oxime K027, in rat plasma. *J Appl Toxicol* 2011;33:18-23. doi: 10.1002/jat.1699
31. Worek F, Baecker M, Thiermann H, Szinicz L, Mast U, Klimmek R, Eyer P. Reappraisal of indications and limitations of oxime therapy in organophosphate poisoning. *Hum Exp Toxicol* 1997;16:466-72. PMID: 9292287
32. Benkő B, Kalász H, Ludányi K, Petroianu G, Kuća K, Darvas F, Tekes K. *In vitro* and *in vivo* metabolisms of K-48. *Anal Bioanal Chem* 2007;389:1243-7. PMID: 17768608
33. Zdarova Karasova J, Zemek F, Kassa J, Kuća K. Entry of oxime K027 into the different parts of rat brain: Comparison with obidoxime and oxime HI-6. *J Appl Biomed* 2014;12:25-9. doi: 10.1016/j.jab.2013.01.001
34. Mercey G, Verdelet T, Saint-André G, Gillon E, Wagner A, Baati R, Jean L, Nachon F, Renard PY. First efficient uncharged reactivators for the dephosphorylation of poisoned

- human acetylcholinesterase. Chem Commun (Camb) 2011;47:5295-7. doi: 10.1039/c1cc10787a
35. Sit RK, Radić Z, Gerardi V, Zhang L, Garcia E, Katalinić M, Amitai G, Kovarik Z, Fokin VV, Sharpless KB, Taylor P. New structural scaffolds for centrally acting oxime reactivators of phosphorylated cholinesterases. J Biol Chem 2011;286:19422-30. doi: 10.1074/jbc.M111.230656
 36. Radić Z, Sit RK, Kovarik Z, Berend S, Garcia E, Zhang L, Amitai G, Green C, Radić B, Fokin VV, Sharpless KB, Taylor P. Refinement of structural leads for centrally acting oxime reactivators of phosphorylated cholinesterases. J Biol Chem 2012;287:11798-809. doi: 10.1074/jbc.M111.333732
 37. Renou J, Mercey G, Verdelet T, Păunescu E, Gillon E, Arboléas M, Loiodice M, Kliachyna M, Baati R, Nachon F, Jean L, Renard P-Y. Syntheses and *in vitro* evaluations of uncharged reactivators for human acetylcholinesterase inhibited by organophosphorus nerve agents. Chem Biol Interact 2013;203:81-4. doi: 10.1016/j.cbi.2012.09.023
 38. Kliachyna M, Santoni G, Nussbaum V, Renou J, Sanson B, Colletier JP, Arboléas M, Loiodice M, Weik M, Jean L, Renard P-Y, Nachon F, Baati R. Design, synthesis and biological evaluation of novel tetrahydroacridine pyridine-aldoxime and -amidoxime hybrids as efficient uncharged reactivators of nerve agent-inhibited human acetylcholinesterase. Eur J Med Chem 2014;78:455-67. doi: 10.1016/j.ejmech.2014.03.044
 39. Kovarik Z, Maček N, Sit RK, Radić Z, Fokin VV, Sharpless KB, Taylor P. Centrally acting oximes in reactivation of tabun-phosphoramidated AChE. Chem Biol Interact 2013;203:77-80. doi: 10.1016/j.cbi.2012.08.019
 40. Garcia EG, Campbell JA, Olson J, Moorad-Doctor D, Morthole IV. Novel oximes as blood-brain barrier penetrating cholinesterase reactivators. Chem Biol Interact 2010;187:199-206. doi: 10.1016/j.cbi.2010.02.033
 41. Wagner S, Kufleitner J, Zensi A, Dadparvar M, Wien S, Bungert J, Vogel T, Worek FG, Kreuter J, von Briesen H. Nanoparticulate transport of oximes over an *in vitro* blood-brain barrier model. PLoS ONE 2010;5(12):e14213. doi: 10.1371/journal.pone.0014213
 42. Shih T-M, Skovira JW, O'Donnell JC, McDonough JH. *In vivo* reactivation by oximes of inhibited blood, brain and peripheral tissue cholinesterase activity following exposure to nerve agents in guinea pigs. Chem Biol Interact 2010;187:207-14. doi: 10.1016/j.cbi.2010.03.006
 43. Voicu V, Rădulescu FŞ, Medvedovici A. Relationships between the antidotal efficacy and structure, PK/PD parameters and bio-relevant molecular descriptors of AChE reactivating oximes: inclusion and integration to biopharmaceutical classification systems. Expert Opin Drug Metab Toxicol 2015;11:95-109. doi: 10.1517/17425255.2015.980813
 44. Katalinić M, Miš K, Pirkmajer S, Grubič Z, Kovarik Z, Marš T. The cholinergic and non-cholinergic effects of organophosphates and oximes in cultured human myoblasts. Chem Biol Interact 2013;203:144-8. doi: 10.1016/j.cbi.2012.09.015
 45. Žunec S, Kopjar N, Želježić D, Kuča K, Musilek K, Lucić Vrdoljak A. *In vivo* evaluation of cholinesterase activity, oxidative stress markers, cyto- and genotoxicity of K048 oxime-a promising antidote against organophosphate poisoning. Basic Clin Pharmacol Toxicol 2014;114:344-51. doi: 10.1111/bcpt.12158
 46. Voicu VA, Bajgar J, Medvedovici A, Radulescu FS, Miron DS. Pharmacokinetics and pharmacodynamics of some oximes and associated therapeutic consequences: a critical review. J Appl Toxicol 2010;30:719-29. doi: 10.1002/jat.1561
 47. Zdarova Karasova J, Pohanka M, Musilek K, Zemek F, Kuca K. Passive diffusion of acetylcholinesterase oxime reactivators through the blood-brain barrier: influence of molecular structure. Toxicol in Vitro 2010;24:1838-44. doi: 10.1016/j.tiv.2010.05.009
 48. Sakurada K, Matsubara K, Shimizu K, Shiono H, Seto Y, Tsuge K, Yoshino M, Sakai I, Mukoyama H, Takatori T. Pralidoxime iodide (2-PAM) penetrates across the blood-brain barrier. Neurochem Res 2003;28:1401-7. doi: 10.1023/A:1024960819430

Translacija učinkovitosti piridinijevih oksima kod trovanja tabunom iz *in vitro* sustava u *in vivo* primjenu

Iako su organofosforni živčani bojni otrovi potpuno zabranjeni za upotrebu, njihova je prisutnost i dalje velik problem, posebice kao kemijsko oružje u terorističkim napadima (poput nedavnih u Siriji). Oksimi koji se danas koriste kao protuotrovi u tretmanu nemaju dostatno djelovanje na reaktivaciju aktivnosti kolinesteraza, glavnih meta djelovanja organofosfornih spojeva. Valja napomenuti kako se klinička testiranja ovih protuotrova rijetko provode zbog svoje iznimne specifičnosti. Tijekom zadnjih desetljeća učinjen je napredak u istraživanju novih učinkovitijih protuotrova, međutim još je uvijek veliki nedostatak u poboljšavanju terapije translacija *in vitro* dobivenih rezultata u *in vivo* primjenu. Ovom studijom ispitali smo mogućnosti ekstrapolacije reaktivacijske učinkovitosti određene za oksimske protuotrove iz *in vitro* u *in vivo* sustav. Naši rezultati pokazuju kako je ova translacija moguća uz detaljno određene kinetičke parametre *in vitro* i uz poznavanje distribucije oksima i vremena cirkulacije u organizmu. Takav rezultat ističe važnost planiranja i farmakokinetičkih istraživanja već u samom početku razvoja protuotrova. Također, poseban naglasak u istraživanju trebalo bi staviti i na poboljšanje tkivo-specifične distribucije oksima u organizmu čime bi se poboljšala cjelokupna terapijska učinkovitost.

KLJUČNE RIJEČI: acetilkolinesteraza; butirilkolinesteraza; farmakokinetika; K048, protuotrovi; specifična aktivnost u tkivima, živčani bojni otrovi