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Comparison of oximes K203 and K027 based on Benchmark dose analysis of rat diaphragmal acetylcholinesterase reactivation

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1. Introduction

Acute human exposure to organophosphorus (OP) group of pesticides presents a well-known health concern due to multiple toxic effects caused primary by a single biochemical event. Phosphorylation of acetylcholinesterase (AChE, EC 3.1.1.7) by OP leads to acetylcholine (ACh) accumulation at postsynaptic sites in cholinergic nerve terminals. Consequently, overstimulation of ACh-receptors results in complex clinical picture of cholinergic crisis, including respiratory muscles paralysis and depression of brain respiratory center, being responsible for lethal outcome [1].

Atropine is a component of the typical antidotal regiment, which competitively antagonize the accumulated acetylcholine at muscarinic but has no impact at nicotinic receptors. Hence, it needs to be accompained by drugs designed to reactivate OP-inhibited AChE - oximes - to attenuate hypercholinergic stimulation not only of muscarinic but also nicotinic receptors, with recovery of respiratory muscle function being the most important for survival in OP intoxication.

However, even six decades after the introduction of pralidoxime [2], the most widely clinically used oxime, and contrary to a huge number of potential oxime reactivators synthesis, their therapeutic efficacy is still much debated [3]. So far, clinical experience as well as experimental studies have shown that currently used oximes are not equally effective in acute poisonings caused by structurally different OPs. Another issue is optimal dosing, which has been identified not only in animal studies but also in patients treated with pralidoxime [4]. Thus, further research should explore different doses and flexibile dosing strategies. In addition, no harmonized experimental strategy exists to evaluate the effects of oximes in the context of dose-response relationship and various methodologies/study designs/protocols have been proposed [5]. Binary system, used in statistical evaluation, results in qualitative differentiation of reactivators that are able to induce an indicative positive response compared to OP-treated group. More specifically, *in vitro* experiments have tested two or three, rarely more, concentrations of oximes vs. control [6–16], *in vivo* studies compared one or two oxime dose vs. control [17–23] and finally, existing data in randomized clinical trials have allowed oxime vs. placebo or higher vs. lower dose comparison [4]. Moreover, safety comparison of oximes, being equaly important as their beneficial activity, becomes adequate only after comparison of equieffective dosages.

We addressed dose-response considerations in *in vivo* study on reactivating effectiveness of two promising experimental oximes K203 and K027 in acute sublethal exposure of rats to OP insecticide model and quantitatively investigated erythrocyte AChE status at six different oxime doses [24]. We have used Benchmark dose (BMD) approach as a comprehensive statistical methodology in toxicological research for analysis of the relationship between dose and effect [25]. Specific advantage of this approach is that it enables determination of effective doses with corresponding effect sizes in biologically/toxicologically interpretable manner and finally enables quantitative comparison of compounds. Quantitative evaluation of oximes in this way improves currently used methodology and allows more stringent comparison of oximes efficacies.

In the manuscript presented, we have extended BMD analysis of oximes K203 and K027 regarding reactivation of diaphragmal AChE, being the neuromuscular synaptic enzyme and thus functionally more important and more appropriate indicator of oximes potential benefit in recovery of compromised respiration compared to widely used surrogate erythrocyte AChE. Thus, primary aim of this study was to model dose-response relationships for oximes K203 and K027 considering rat diaphragm AChE reactivation, secondary to quantify their effective doses with corresponding size of diaphragm AChE-reactivating effect and the third to quantitatively compare them.

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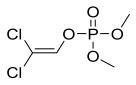
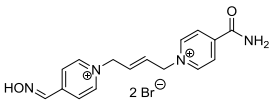
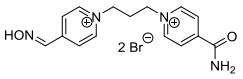
Table 1
Scheme of study design.

	Dichlorvos (DDVP)	Oxime K203	Oxime K027
Chemical structure			
Dose	75% LD_{50}[a]	1.25/2.5/5/25/50% LD_{50}[a]	
Route	*subcutaneously*	*intramuscularly*	
Endpoint	60 min diaphragmal AChE activity (male *Wistar* rat)		

[a] LD_{50} were previously determined [18].

2. Materials and methods

2.1. Chemicals

Oxime K203 (4-carbamoyl-1-[(2*E*)-4-{4-hydroxyiminomethylpyridinium-1-yl}but-2-en-1-yl]pyridinium dibromide; Mw = 458.15 g/mol) and oxime K027 (4-carbamoyl-1-(3-{4-hydroxyiminomethylpyridinium-1-yl}propyl)pyridinium dibromide; Mw = 446.14 g/mol) were synthesized at the Department of Chemistry, Faculty of Science, University of Hradec Kralove (Czech Republic). Their purity (> 98%) was controlled using a HPLC technique. Dichlorvos (DDVP, *O,O*-dimethyl-*O*-2,2-dichlorovinyl phosphate; Mw = 220.98 g/mol; purity 98.8%) was gifted by Chemical Agrosava d. o.o. (Belgrade, Serbia). Chemical structures of tested oximes and DDVP are shown in Table 1.

Required concentrations of oximes were prepared by *ex tempore* dissolution in distilled water. Isopropyl alcohol was used as a stock solvent for DDVP whereas dilutions were made *ex tempore* in distilled water. Commercially obtained all other chemicals and reagents were of analytical grade.

2.2. Animals

Male outbred Wistar rats (180–200 g), obtained from the Military Medical Academy (Belgrade, Serbia), were used as experimental model. Animal housing and handling [24] was carried out in accordance with the Animal Welfare Act of the Republic of Serbia (Official Gazette of the Republic of Serbia No. 41/2009) and Directive 2010/63/EU on the Protection of Animals Used for Scientific Purposes, while the experimental protocol was approved by Ethical Committee on Animal Experimentation of the University of Belgrade, Faculty of Pharmacy (Serbia, No. 323-07-00363/2017-05). Animals were exposed to 12/12 h light/dark cycle; food and tap water were available *ad libitum*.

2.3. Study protocol

The rationale of optimal study design for assessing the BMDs for experimental oximes considering OP-inhibited AChE reactivation has been reported in Antonijevic et al. [24] and the scheme is presented in Table 1. Briefly, the study consisted of six dose groups (5 rats per dose) for each oxime tested. The dose of 30 μmol/kg DDVP, which corresponds to 75% of median lethal dose (LD_{50}) in unprotected animals [18], was given subcutaneously (s.c) over the flank to rats. Oxime K203 at doses 0, 9, 17, 35, 179 or 358 μmol/kg or oxime K027 at doses 0, 31, 63, 126, 632 or 1264 μmol/kg (Table 1) was given intramuscularly (i.m) into the back of the thigh immediately after OP. Administered volume of tested compounds was 1 mL/kg body weight (b.w). Activity of AChE was measured 60 min after the oxime treatment [18,26].

2.4. Processing of rat diaphragm and AChE activity assay

Dissected diaphragms were rinsed in cold saline solution (0.9% NaCl), blotted, weighed and homogenized in saline (1:19, w/v) using homogenizer T10 basic ULTRA-TURRAX (IKA, Germany). The homogenates were then centrifuged at 3000 g for 15 min and AChE activity was measured in supernatants by modified Ellman's spectrophotometric method [27] with 1 mM acetylthiocholine iodide (Acros Organics, USA) in a phosphate buffer (pH = 7.4) at 25 °C, using Cary60 UV–Vis spectrophotometer (λ = 410 nm) equipped with a Cary single cell Peltier for temperature-controlled analysis (Agilent Technologies, USA).

2.5. Data analysis

Freely available PROAST software version 65.5 (Dutch National Institute for Public Health and the Environment (RIVM)) was used for analysis of dose-response relationship in the R computing environment (version 3.4.2). Hill and exponential models, for continuous data type, along with Akaike information criterion (AIC) were applied to obtain the best model of dose-response [28].

In both model families, with oxime set as covariate, y denotes the response and x the dose. Result of the fitted model are parameters: *var* - within-group variation; a - the background response (response at $x = 0$); b - potency of the chemical; c - maximum response and parameter d - steepness of the curve [29].

Estimation of critical effect dose (CED) with its confidence interval (CI, bounded from lower, CEDL to upper, CEDU limit) was used in order to ensure robust potency ranking of compounds (significant if confidence intervals do not overlap) within endpoint [30–32] and to calculate precision of estimated CED (CEDU-to-CEDL ratio) [33].

Endpoint specific critical effect size (CES) values, defined as a change in response relative to the background, were obtained using statistical analysis established by Wout Slob [34].

In order to quantify the potency difference, CED of oxime K203 was treated as a reference value, meaning that CED of K027 was relative to the corresponding K203 value, expressed as relative potency factor (RPF).

Finally, CED ratio, defined as CED_{large} divided by $CED_{medium,}$ was calculated in order to measure a rate of dose-response change [29].

3. Results

3.1. Model of oxime dose-AChE activity relationship in rat diaphragm

In one-hour period of observation after the treatment, we did not record lethal effect in DDVP- or oxime-treated group of animals.

The best output of the relationship between increasing doses and corresponding diaphragmal AChE activity, for both oximes, was model 5, regardless the models used (Fig. 1A). However, improper fit ($AIC_{min} > AIC_{full} + 2$) was found: $AIC_{exp} = -46.02$, $AIC_{Hill} = -43.6$,

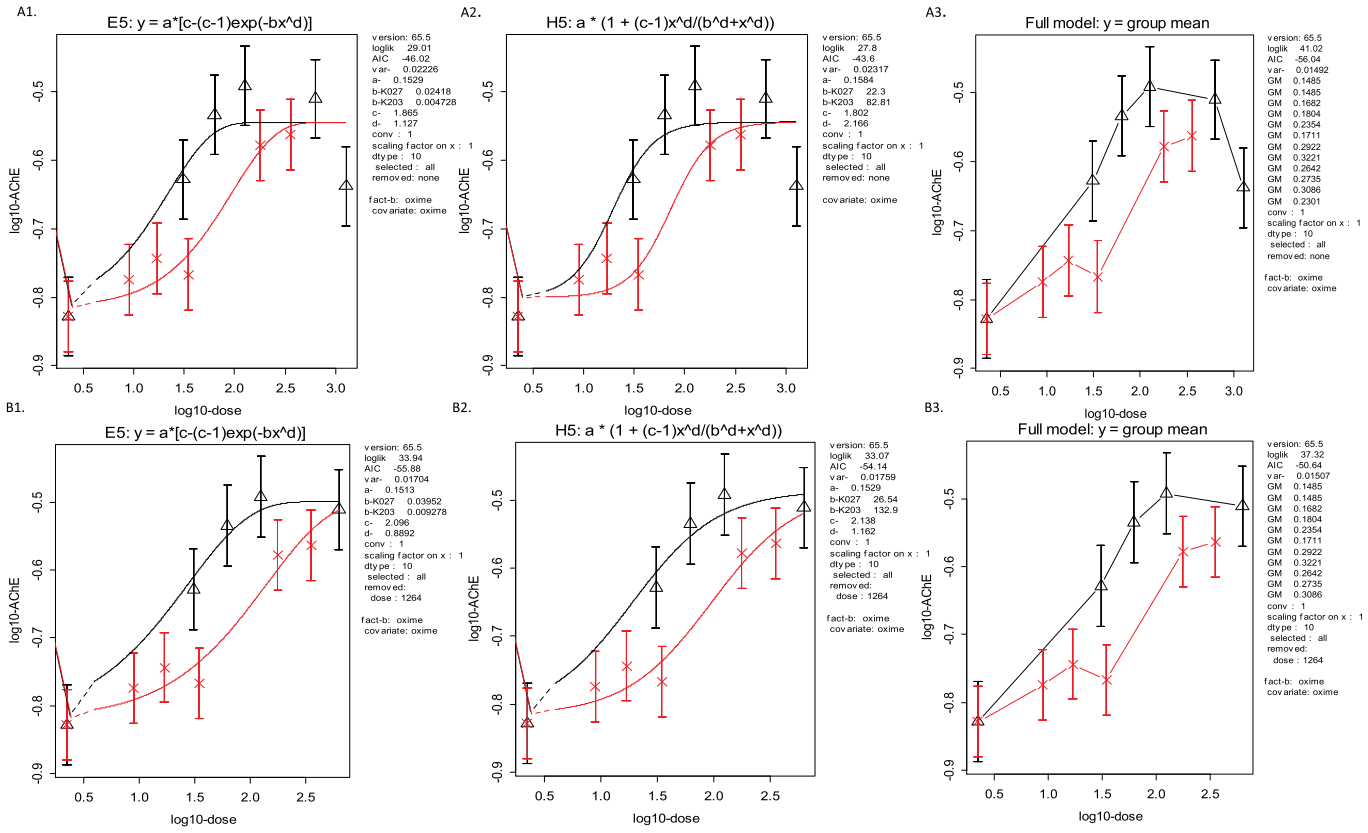


Fig. 1. AChE activity in rat diaphragm as a function of dose related to oxime K027 (triangles) and oxime K203 (crosses), reactivators of DDVP-inhibited AChE (response at dose zero). The fitted curves represent exponential (1), Hill (2) and full (3) model before (A) and after (B) omitting the highest dose group of oxime K027, with the values of parameters *a*-response at dose zero, *b*-potency, *c*-maximum response and *d*-steepness shown to the right of each plot. The vertical bars represent 90% confidence intervals of geometric mean responses.

$AIC_{full} = -56.04$. Assuming that the highest oxime K027 dose does not fit into the curve properly, the data were reanalyzed and the highest dose was excluded from the data set. Reanalysis resulted in model 5, which was in compliance with the above mentioned criterion: $AIC_{exp} = -55.88$, $AIC_{Hill} = -54.14$, $AIC_{full} = -50.64$ (Fig. 1B). Significant difference in parameter *b* (i.e. exponential: *b*-K203 = 0.009278 and *b*-K027 = 0.03952, Fig. 1B1) showing the right-shifted position of the K203 curve compared to K027, has indicated the difference in the potency of two oximes in reactivation of diaphragmal AChE. Further extension of analysis in terms of oxime-dependent parameter *c* did not result in significant AIC-decrease ($AIC_{exp} = -55.1$). Here in addition, the obtained 90%-CIs for parameter *c* (i.e. exponential: *c*-K203 1.62–2.81, *c*-K027 1.88–2.32) were overlapped between oximes, which would not cause significantly different estimates of BMRs and consequently BMDs. Therefore, the assumption of oxime-dependent maximum AChE reactivation was abandoned in further analysis.

Having in mind that there was no substantial difference between the results of the two models, as well as practical reason of easier following the results, the exponential model results are only given in the text, while Figures and Tables contain the results of both models.

3.2. Critical effect doses of oximes K203 and K027

Oxime-induced recovery of inhibited AChE at maximum size was 2.1-fold (Fig. 1B), given as percent change resulted in (2.1-1)*100 = 110%. Further on, applying the statistical method based on a general theory of effect size [34] resulted in three levels of effect sizes as follows:

"Large" effect size = $M^{1/2} = 2.1^{1/2} = 1.449$-fold increase or 45%,

"Medium" effect size = $M^{1/4} = 2.1^{1/4} = 1.204$-fold increase or 20%,

"Small" effect size = $M^{1/8} = 2.1^{1/8} = 1.097$-fold increase or 10%.

Related critical effect doses (CED) were for oxime K027 $CED_{45} = 18$, $CED_{20} = 6$ and $CED_{10} = 3$ μmol/kg and for oxime K203 $CED_{45} = 94$, $CED_{20} = 32$ and $CED_{10} = 14$ μmol/kg (Table 2). However, confidence intervals of CED for oximes did not overlap only at the large scale reactivation (45%), resulting in statistically supportable higher ability of oxime K027 to antagonize toxicity of DDVP than oxime K203 (Fig. 2 A1, B1, C1). Reactivation of diaphragmal AChE obtained by oxime K027 was 5.103 (CI = 3.13, 9.02) times higher than reactivation

Table 2
Critical effect doses (CED, μmol/kg) of oximes K027 and K203 with 90%-confidence intervals (lower, CEDL and upper, CEDU bound) and uncertainty measure (CEDU/CEDL ratio) at three levels of critical effect size (CES) concerning reactivation of DDVP-inhibited AChE in rat diaphragm.

		Exponential m5-*b*		Hill m5-*b*	
		K027	K203	K027	K203
CES = 45%	CED	18	94	18	92
	CEDL	9	54	8	51
	CEDU	31	156	33	159
	CEDU/CEDL	3.4	2.9	4.1	3.1
CES = 20%	CED	6	32	7	35
	CEDL	2	14	2	14
	CEDU	14	68	18	85
	CEDU/CEDL	7.0	4.9	9.0	6.1
CES = 10%	CED	3	14	4	18
	CEDL	1	4	1	5
	CEDU	8	38	13	59
	CEDU/CEDL	8.0	9.5	13.0	11.8

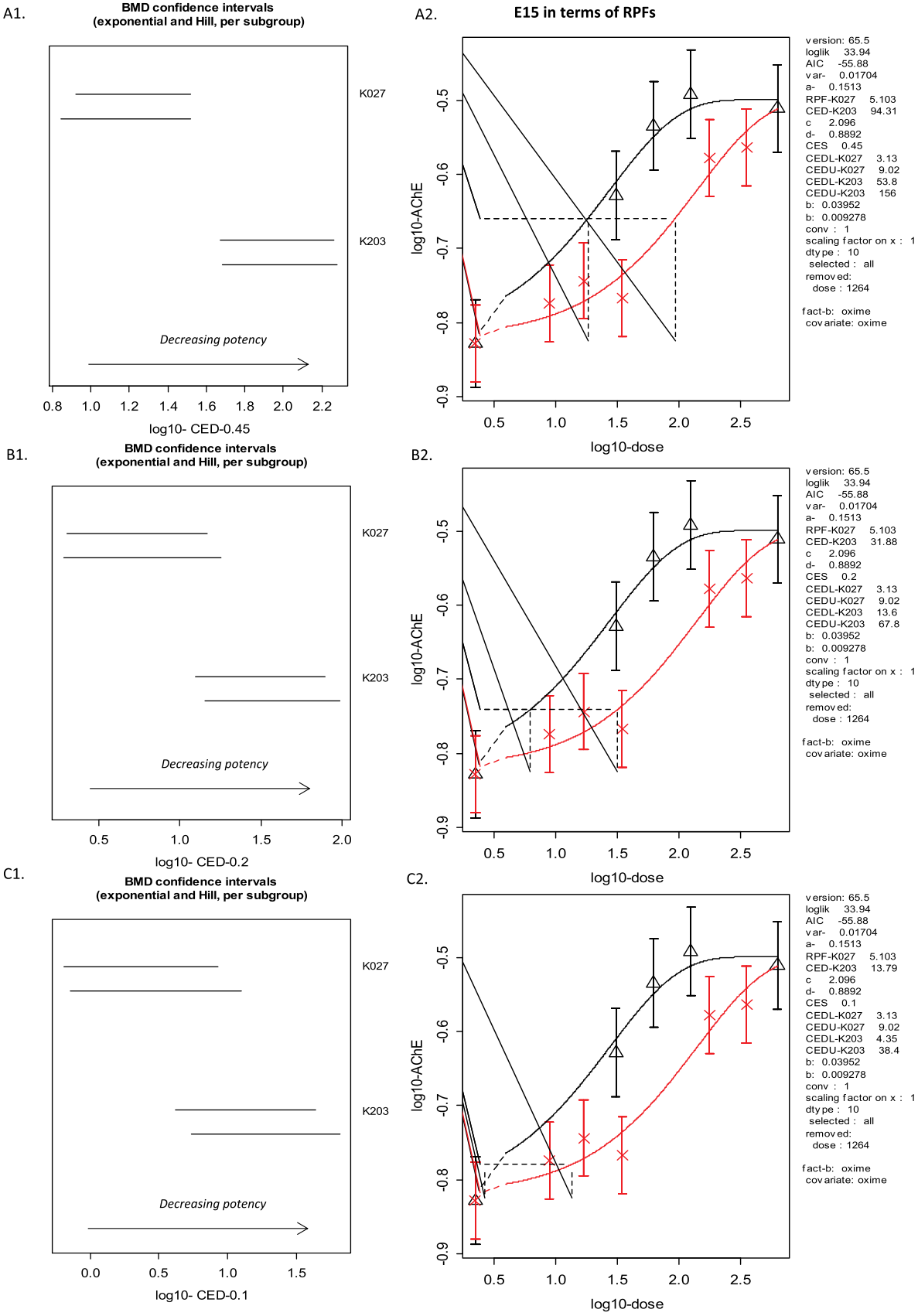


Fig. 2. AChE activity in rat diaphragm as a function of dose related to oxime K027 (triangles) and oxime K203 (crosses), reactivators of DDVP-inhibited AChE (response at dose zero). Left plots (1) represent 90% confidence intervals of critical effect dose (CED) per oxime at three levels of critical effect sizes CES = 45% (A1), CES = 20% (B1) and CES = 10% (C1), respectively. The curves on right plots (2) reflect the fitted exponential model in terms of relative potency factor (RPF) (RPF > 1, higher potency, lower CED) at three levels of CES (A2, B2, C2). Values of RPF-K027 (arbitrary unit), CED-K203 (μmol/kg) along with 90% confidence intervals as well as model parameters *a*-response at dose zero, *b*-potency, *c*-maximum response and *d*-steepness, are shown to the right of each plot. The vertical bars represent 90% confidence intervals of geometric mean responses.

produced by oxime K203 (CED_{K203} = RPF × CED_{K027}) (lower CED) (Fig. 2A-C2). By effect size increasing, higher precision of CED estimate, given as ratio CEDU/CEDL, was observed, i.e. ratios were 3.4 of oxime K027 and 2.9 of oxime K203, proving that dose-response curves were appropriate (Table 2).

4. Discussion

Dose-response studies on experimental oximes, which would contribute to the establishment of effective doses, valuable for defining optimal dosage regimens in OP-intoxications, are lacking.

Therefore, we measured *in vivo* reactivating effect of two promising experimental oximes on AChE inhibited by DDVP, used as dimethyl OP-structural model with oxon moiety that provides direct inhibition of AChE, in the range of doses 1.25–50% LD_{50} (i.m, rat). In terms of data analysis, BMD approach was used as comprehensive and highly informative approach for dose-response modeling, for the first time in evaluation of reactivators efficacy, according to our best knowledge. However, BMD approach has been developed for the establishment of the relevant point of departure in chemical risk assessment, including OP pesticides with AChE activity as specific endpoint [35]. Additionally, taking into consideration 3Rs (Replacement, Reduction and Refinement) principles, this approach represents important step forward in *in vivo* experimentation, in particular reduction [33,36].

After we quantified a dose-related reactivation of surrogate marker - erythrocyte AChE - for oximes K203 and K027 in our recent paper [24], in this work we have evaluated that relationship for diaphragmal AChE as the main therapeutic benefit of oximes is recovery of neuromuscular transmission.

After administration of the top oxime K027 dose activity of diaphragmal AChE has decreased, which was highly consistent with our findings in erythrocytes [24]. This finding can be ascribed to the toxicity phenomenon of relatively high oxime K027 dose (50% LD_{50}). Oximes at high doses may inhibit intact AChE according to their intrinsic affinity [14]. Secondly, formed phosphylated-oxime may inhibit newly-reactivated AChE [37–39] and more specifically, it has been shown that oxime K027 impaired hepatic excretory function in rats at exactly dose 50% LD_{50} (i.m) [40].

In diaphragm tissue compared to erythrocytes lower maximal size of AChE reactivation by oximes was observed (110% vs. 170%, respectively). However, theory applied in this work for assessment of meaningful effect sizes for continuous toxicological endpoints [34] has allowed calculation of equivalent effect sizes among diaphragm tissue and erythrocytes (large CES = 45% vs. 58%, respectivey) and consequently equieffective doses of oximes. Lower maximal level of AChE reactivation observed in diaphragm may be explained by expected limited bioavailability to intracellular fraction of muscle AChE due to quaternary structure of oximes, which is in line with very low plasma:muscle fraction of oximes K027 and K203 (~2 vs. 3.5%) found in experimental pig [41]. Moreover, external (surface) AChE activity amounts ~20% of total homogenate activity [42]. However, when the surface AChE inhibition amounted less than 35%, along with internal AChE inhibition in a range of 80–90%, normal twitch response of the rat diaphragm was found *in vitro* [42].

Consistent with maximal effect size, lower steepness of the dose-response curves was observed in diaphragm compared to erythrocytes and quantitatively expressed as CED ratio, this means that higher increase of oxime dose was required to produce the same increase in AChE reactivation in diaphragm as in erythrocytes (CED ratio = 3 vs. 2, respectively, Table 3). Given that no biological mechanism is reflected by fitted models in dose-response modeling [43], involvement of direct antinicotinic and antimuscarinic effects of oximes K027 and K203 [44,45] at the neuromuscular junction, apart from OP-inhibited AChE reactivation effect, might be expected to cause the lowering of the curves steepness. This, further, contributed to broader CIs of equieffective CEDs of oximes in diaphragm compared to erythrocytes (Fig. 3),

Table 3
Critical effect dose (CED) ratios for erythrocytes and diaphragm tissue of rats concerning reactivation of DDVP-inhibited AChE by oximes K027 and K203.

	Erythrocytes[a]		Diaphragm	
	K027	K203	K027	K203
CED_{large} (μmol/kg)	52	100	18	94
CED_{medium} (μmol/kg)	24	47	6	32
CED ratio	2	2	3	3

[a] Erythrocytes CEDs were previously determined [24].

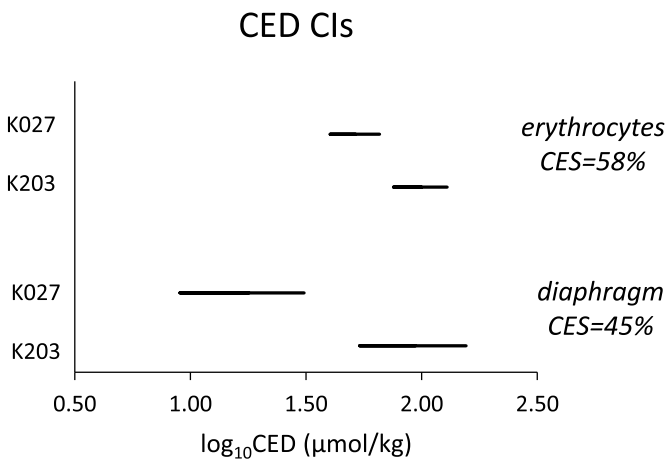


Fig. 3. Potency of K-oximes in reactivating diaphragmal and erythrocytes AChE inhibited by DDVP in rats. Horizontal lines represent two-sided 90% confidence intervals (CIs) of critical effect doses (CEDs) for oximes at log10-scale. The BMD analysis was done per tissue with oxime as covariate and the underlying exponential dose-response curves are presented in Fig. 1B (this paper) for diaphragm and Supp. Fig. 2 (ref. [24]) for erythrocytes.

but even apart from that, as Fig. 3 indicates, sensitivity of diaphragm was found to be higher in terms of oxime K027, while difference in sensitivity of tissues for oxime K203 could not be established due to overlapping CIs of equieffective CEDs.

Oxime K027 was more potent in reactivating DDVP-inhibited AChE in rat diaphragm compared to oxime K203 (CED_{45}-K027 = 18 μmol/kg vs. CED_{45}-K203 = 94 μmol/kg), which is consistent with their potency ranking observed in erythrocytes. Generally, although oximes K027 and K203 show similar i. m. apsorption kinetics (c_{max} and t_{max}) in experimental rats and pigs [41,46], lower equieffective doses of oxime K027 compared to oxime K203 found in our study could be partially explained by its ~3-fold lower protein binding potency found in human serum albumin binding study [46] and ~2-fold lower plasma:urine ratio and elimination via kidney found in experimental pigs (i.m) [41], implying its higher pharmacologically active concentrations *in vivo*. Moreover, relative potency factor of oxime K027 was higher in diaphragm compared to erythrocytes (RPF = 5.103 vs. 1.903, respectively).

Functional equivalent to diaphragmal AChE activity is neuromuscular transmission (NMT) [47] and it has been shown *ex vivo* that impaired muscle force generation correlates with diaphragmal AChE activity levels lower than ~25% of normal value in the presence of paraoxon [48]. In addition, once the critical level is reached, the complete tetanic tension has been observed *ex vivo* even a very small further reduction in an enzyme activity existed [49]. Traditionally, activity of AChE is expressed as % of the enzyme activity measured in untreated control group. Effect size of 45% obtained in our study presents the amount of reactivated AChE expressed as % of enzyme activity in DDVP-treated group of animals. Hence, expressing CES of 45% in traditional way, would result in 28% AChE activity of control level, and

thus, being higher than abovementioned critcal level, we could expect normal muscle function at doses of CED_{45}-K027 = 18 μmol/kg and CED_{45}-K203 = 94 μmol/kg.

Finally, in order to evaluate safety-efficacy profile for oximes K027 and K203, based on our results, we have chosen to compare the upper bound of estimated therapeutic doses ($CEDU_{45}$) and the highest tested dose (50% LD_{50}), identified as the dose of oxime K027 at which no further beneficial effect is expected, as follows:

Therapeutic index, TI = 50%Action: Delete Edit: , TI = 50% < correction > < /correction > LD50/CEDU4 LD50/CEDU45.

TI_{K203} = 358 μmol/kg/156 μmol/kg ≈ 2 vs. TI_{K027} = 1264 μmol/kg/31 μmol/kg ≈ 41.

Oxime K027 has broader TI for diaphragmal AChE reactivation compared to oxime K203, as already argued for erythrocyte AChE reactivation [24], implies consistent and promising finding in terms of possibility of higher cumulative dose in repeated intermittent therapeutic regimens or bolus dose plus continuous infusion [50].

In conclusion, evaluated relationships between dose and response for oxime K203 and oxime K027 regarding *in vivo* ability to reactivate diaphragmal AChE inhibited by DDVP in rats, were described by (i.e.) exponential model m5-*b* ($y = a\ [c\text{-}(c\text{-}1)\exp\ (\text{-}bx^d)]$), $a = 0.1513$, $b_{K027} = 0.03952$, $b_{K203} = 0.009278$, $c = 2.096$ and $d = 0.8892$.

Equieffective doses of oximes were estimated to be 18 μmol/kg (CI: 9, 31 μmol/kg) for oxime K027 and 94 μmol/kg (CI: 54, 156 μmol/kg) for oxime K203, and were significantly different at 45% effect size resulting in 5 times higher potency of oxime K027.

In addition, taken together with our recent findings on erythrocytes AChE activity decrease trend at top tested dose of oxime K027, cosistent result on diaphragm AChE activity indicate that maximal dose in its further *in vivo* testing should not exceed the amount of 632 μmol/kg (282 mg/kg).

Quantification of equieffective doses of experimental and clinically used oximes by the combined BMD covariate method would improve their comparison, thus contributing in defining therapeutic dosing strategies and finally, increase human relevance of animal data on oximes efficacies.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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