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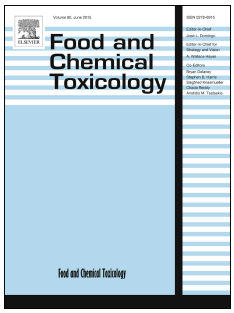

DOSE-RESPONSE modeling of reactivating potency of oximes K027 and K203 against a direct acetylcholinesterase inhibitor in rat erythrocytes

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DOSE-RESPONSE MODELING OF REACTIVATING POTENCY OF OXIMES K027 AND K203 AGAINST A DIRECT ACETYLCHOLINESTERASE INHIBITOR IN RAT ERYTHROCYTES

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Inhibition of acethylcholinesterase (AChE) as a key molecular event induced by organophosphate (OP) pesticides and nerve agents presents a human health concern. In efficacy testing of experimental oximes, potential antidotes in OP poisoning, reactivation of OP-inhibited AChE is used as specific endpoint. However, according to our best knowledge, so far oximes have not been quantitatively evaluated by comprehensive benchmark dose (BMD) approach, that would improve both identification and quantification of the effect and allow more rigorous comparison of efficacies. Thus, we have examined *in vivo* dose-response relationship for two promising experimental oximes, K203 and K027, concerning reactivation of erythrocyte AChE inhibited by dichlorvos (DDVP). Groups of Wistar rats were treated with six different doses of oximes (i.m) immediately after DDVP challenge (s.c) and AChE was measured 60 min later. Dose-response modeling was done by PROAST software 65.5 (RIVM, The Nederlands). BMD-covariate method resulted in four-parameter model from both exponential and Hill model families as the best estimate of relationship between AChE activity and oxime dose, with potency parameter being oxime-dependent. Oxime K027 was shown to be 1.929-fold more potent considering that 58% increase in AChE activity was achived with the dose BMD_{58}-K027 = 52 µmol/kg in contrast to BMD_{58}-K203 = 100 µmol/kg.

Keywords: dichlorvos, K027 vs K203, rat erythrocytes, benchmark dose, PROAST

1. Introduction

Oxime reactivators are chemical compounds used as causal antidotes for the treatment of human poisoning with organophosphorus compounds (OP). Human exposure to OPs may arise from the use of OP nerve agents in terrorist attacks, from an occupational exposure to OP pesticides or from the suicidal self-poisonings. The principal mechanism of action of oximes is reactivation of OP-inhibited enzyme acetylcholinesterase (AChE, EC 3.1.1.7) and consequently recovery of the enzyme's physiological function (Hobbiger, 1963). Successful reactivation can terminate overstimulation of muscarinic and nicotinic receptors by acetylcholine (ACh) and thus prevent neurotoxicity (Mileson et al., 1998; Pope et al., 2005). However, experimental results and clinical findings have demonstrated insufficient and/or unequal therapeutic value of structurally-different oximes against structurally-different OPs (Antonijevic and Stojiljkovic, 2007; Eyer, 2003; Worek et al., 2016). Thus, the therapy of human OP poisoning still presents a demanding issue and the search for oxime with higher efficacy and/or a broader spectrum of activity compared to clinically used oximes is a challenging task. Moreover, heterogeneity of used experimental and epidemiological methodologies makes a rigorous evaluation and comparison of efficacies of both clinically used and promising experimental oximes difficult (Worek and Thiermann, 2013).

Nevertheless, traditionally in the *in vivo* testing, effects of oximes have been evaluated at only one or two different doses in the same experimental protocol and the common way of analysing dose-response data has been statistical testing of each dose group against the controls. That approach enables qualitative evaluation of oximes efficacies in order to differentiate compounds that are able to elicit a significant positive response. However, quantitative evaluation of oximes would improve both identification and quantification of the effect and allow more rigorous comparison of their efficacies. Quantitative evaluation of dose-response relationship as a whole has been enabled by Benchmark Dose (BMD)

methodology introduced by (Crump 1984).. The BMD approach involves statistical analysis of the relationship between dose and response to define the dose (BMD) required to elicit a pre-specified change in response (benchmark response, BMR). The BMD is estimated from the complete dose-response dataset by fitting dose-response models. Analysing historical ("available") data, (Slob and Setzer, 2014) have shown that large number of toxicological endpoints (both *in vitro* and *in vivo*), including AChE activity, can be adequately described by 4-parameter exponential or Hill model family. Statistical uncertainty in estimated BMD value is presented as the confidence interval around the BMD (lower, BMDL and upper, BMDU limit). At present, BMD approach is increasingly used for the evaluation of toxicity dose-response data and calculation of BMD as a point of departure for deriving human exposure limits in human health risk assessment **(EFSA, 2017, 2009; Slob, 2002)**. Moreover, BMD approach implies a considerable step forward from the perspective of the principles of the 3Rs (Replacement, Reduction and Refinement) in animal experimentation, in particular in R of reduction, because all animals in experiment contribute to BMD calculation, alowing getting more information from the same number of animals (Slob, 2014a, 2014b).

Thus, the specific aim of this sudy was to quantitatively evaluate and compare efficacies of two promising experimental oximes, K203 and K027, considering reactivation of OP-inhibited AChE using comperhensive BMD approach.

We performed *in vivo* study on experimental rats acutely exposed to OP insecticide dichlorvos (DDVP), used as dimethyl OP-structural model compound with oxon moiety that provides direct inhibition of AChE. Towards DDVP-inhibited erythrocyte AChE we confronted oximes K203 and K027, with characterized *in vitro* reactivating and *in vivo* therapeutic and reactivating potencies against different OPs (Antonijevic et al., 2018, 2016; Arshad et al., 2018; Berend et al., 2012; Jun et al., 2010; Kassa et al., 2008; Kovarik et al., 2009; Kuca et al., 2018, 2010; Lorke et al., 2008; Musilek et al., 2010, 2008, 2007a, 2007b; Musilova et al.,

2009; Petroianu et al., 2007a, 2007b, 2006b, 2006a). In contrast to published *in vivo* experimental protocols, that tested one or rarely two oxime doses, here we have tested six different doses of oximes K203 and K027, with primary aim to model their dose-response relationships, secondary to quantify size of the AChE-reactivating effect with corresponding effective doses and the third to quantitatively compare them.

We have applied recently developed advanced BMD methodology – combined BMD covariate method – that permits simultaneous analyses of multiple dose-response datasets for a shared endpoint (Slob, 2002; Slob and Setzer, 2014), because it has been shown that it improves the precision of each individual BMD estimate (Slob and Setzer, 2014; Wills et al., 2016a, 2016b).

Moreover, we have applied estimate of BMD confidence intervals, being recently shown as robust way for compound potency ranking within and/or across endpoints (Long et al., 2018; Wills et al., 2017, 2016a, 2016b).

We have also assessed and discussed the importance of appropriately defining benchmark response values (BMRs) in evaluation of oximes as reactivators of OP-inhibited AChE.

2. Materials and methods

2.1. *Animals*

All experimental procedures were approved by the Ethical Committee on Animal Experimentation of the University of Belgrade, Faculty of Pharmacy (Serbia, No. 323-07-00363/2017-05) and were 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. Male outbred Wistar rats were purchased from the Military Medical Academy (Belgrade, Serbia). They were housed in polycarbonate cages (425×266×185 mm^3, up to five rats per cage, randomly

selected) in access- and climate-controlled rooms for seven days of acclimatization before the experimental procedure. The room temperature was 22 ± 2 °C, the relative humidity 55 ± 10%, the illumination 120 lx and the 12/12 h light/dark cycle (light on at 6:00 h). Food and tap water were available *ad libitum*. The food was standard maintenance chow for rats purchased from The Veterinary Institute Subotica (Subotica, Serbia). Experimental procedure was carried out on eight weeks old rats weighing 180-200 g.

2.2. *Chemicals*

Dichlorvos (DDVP, *O,O*-dimethyl-*O*-2,2-dichlorovinyl phosphate, CAS number: 62-73-7) was obtained as a gift from Chemical Agrosava d.o.o. (Belgrade, Serbia) and was 98.8% pure. Oxime K203 (4-carbamoyl-1-[(2*E*)-4-{4-hydroxyiminomethylpyridinium-1-yl}but-2-en-1-yl]pyridinium dibromide) and K027 (4-carbamoyl-1-(3-{4-hydroxyiminomethylpyridinium-1-yl}propyl)pyridinium dibromide) were synthesized at the Department of Chemistry, Faculty of Science, University of Hradec Kralove (Czech Republic) and they were more than 98% pure. Their purity was analysed using a HPLC technique. Chemical structures, names, and molecular weights of OP insecticide DDVP and tested oximes are presented in Table 1.

A stock solution of DDVP was prepared in isopropyl alcohol and further dilutions were made in distilled water *ex tempore*. Oximes were dissolved in distilled water and diluted to the required concentration *ex tempore*. All other chemicals and reagents of analytical grade were obtained commercially.

Table 1

2.3. *Study design and experimental procedure*

Study design for the purpose of dose-response analysis for oximes considering reactivation of DDVP-inhibited AChE was based on following: 1) optimal study design for the estimation

BMD in continuous endpoints with large coefficient of variation (CV≥18%) was found to be six dose groups (Slob et al., 2005); 2) analysis of 32 *in vivo* studies derived standard deviation of 18.6% in OP-inhibited AChE as an endpoint (Slob, 2017; Slob and Setzer, 2014). Thus, we have set six dose groups (including zero dose) for each oxime. Rats were divided into 11 experimental groups (5 per group) as follows: group 1 = DDVP; groups 2-6 = DDVP+K203 and groups 7-11 = DDVP+K027. Rats were injected subcutaneously (s.c) over the flank with DDVP at a dose 30 µmol/kg, which had been reported to cause less than 50% lethality in unprotected animals (Antonijevic et al., 2016). Immediately after DDVP injection the rats were injected with oxime dose (Table 1) intramuscularly (i.m), into the back of the thigh. The highest dose of tested oxims had been reported to cause less than 50% lethality (Antonijevic et al., 2016). Both administrations were done via a 27G needle/syringe system. All compounds were administrated at a volume of 1 mL/kg body weight (b.w.). Treatments were performed in the mornings between 8 and 9 a.m. The rats were humanely euthanized 60 min after the treatment, expecting at that time maximal severity of intoxication (Antonijevic et al., 2016; Duarte et al., 2012).

2.4. *Processing of the tissue and biochemical assay*

Blood samples were collected into heparinized syringes by intracardial punction and were analysed immediately after specimen collection. Erythrocytes were separated by centrifugation at 3000 xg for 15 min, washed three times in saline solution (0.9% NaCl) and hemolysed in distilled water (1:600, v/v). Activity of AChE was determined by the modified spectrophotometric method of (Ellman et al., 1961) using acetylthiocholine iodide (Acros Organics, USA) as substrate in a final concentration of 1.75 mM (stock solution 35 mM),

phosphate buffer (pH=7.4) and temperature 25 °C. Absorbance was read at 412 nm using Cary60 UV-Vis spectrophotometer with a Cary single cell Peltier for temperature control (Agilent Technologies, USA). AChE activity was expressed in unites µmol of hydrolyzed acetylthiocholine iodide/min per mL erythrocytes.

2.5. *Dose-response data analysis*

BMD analysis, statistical analysis of the dose-response data, was conducted using PROAST software version 65.5 in the R computing environment (Dutch National Institute for Public Health and the Environment (RIVM)). PROAST v65.5 is available for free download at http://www.proast.nl. As recommended by the European Food Safety Authority (EFSA) for continuous type of data, the analysis was done using both the exponential and the Hill model families (EFSA, 2017, 2009). PROAST uses Akaike information criterion (AIC) to arrive to the optimal model within model family, where the model with the lowest AIC is selected (EFSA, 2017). Briefly, a dose-related trend is conifrmed if AIC is lower than the AIC of the specific null model – 2 units ($AIC<AIC_{null}-2$). The null model expresses the situation that there is no dose-related trend, i.e. it is a horizontal line ($y=a$), and it tends to show the largest AIC. Further, the lowest AIC should not exceed the AIC of the specific full model by more than 2 units ($AIC<AIC_{full}+2$). The full model describes the dose–response relationship simply by the observed (mean) responses at the tested doses, without assuming any specific dose–response and it tends to show the smallest AIC.

In both model families, y denotes the response, x the dose, while *var*, a, b, c and d are parameters to be estimated by fitting the model with same interpretation in both model families. Parameter *var* reflects within-group variation, a reflects the background response (response at x=0, vertical scaling parameter), parameter b relates to the potency of the chemical (horizontal scaling parameter, in the exponential model, $b^{1/d}$ is directly proportional to potency, while in the Hill, $1/b$ is directly proportional to potency), parameter c reflects

maximum response (maximum fold-change relative to the background response, shape parameter) and parameter *d* reflects the steepness of the curve (shape parameter) (Slob and Setzer, 2014). The combined BMD covariate approach was used, where "oxime" was set as covariate, factor discriminating the dose-response subgroupings. The combined BMD covariate approach, identifies the model parameters that are significantly different for each subgroup, alongside those that can be considered constants across subgroups (Slob, 2002). Parameters that were not significantly different were kept the same for both tested oximes in analysis. This combined approach was recently shown to improve the precision of each individual BMD estimate as all dose-response datasets included in analysis contribute to individual BMD estimate (Slob and Setzer, 2014; Wills et al., 2016a, 2016b).

Benchmark dose (BMD) is derived from a fitted model as a result of pre-specified change (increase or decrease) in the response (benchmark response, BMR). In PROAST output for continious data, terms critical effect size (CES) and critical effect dose (CED) are used instead BMR and BMD, respectively (Slob and Pieters, 1998) and thus will be employed in the paper. The CEDL and CEDU values represent the lower and upper 90% (default value in PROAST software) CED confidence intervals, respectively, thus the CEDU-to-CEDL ratio defines the CED estimate precision (Slob, 2014b). PROAST software calculates confidence interval bounds by the profile likelihood method (also called the likelihood ratio method). Confidence interval plots were employed to visually compare differences in potency, in order to take CED estimation precision into account (Bemis et al., 2016; Wills et al., 2017, 2016a, 2016b). Dose-response relationships across subgroupings (e.g. oximes) can only be termed significantly different where confidence intervals do not overlap.

CES is defined as a percent change in mean response relative to the background response. Endpoint specific CES values were calculated on the basis of values of fitted models

parameters *c* and *var*, applying the statistical method established by (Slob, 2017) and built on a general theory of effect size given in the same framework.

An additional model, equivalent to exponential, where the different CEDs (related to different subgroups) are expressed as relative potency factors (RPFs) relative to the CED of one of the subgroups (e.g. the reference chemical) was also applied. In our analysis, CED of oxime K027 was expressed as RPF relative to the CED of oxime K203, which was set as a reference compound. This model allowed calculation of confidence intervals for the RPF.

Shematic ilustration of the BMD approach is presented in Figure 1.

3. Results

No lethal oucome was observed during the 60 min period between dosing and euthanasia in DDVP-treated group nor any oxime-treated group of animals. However, DDVP-treated animals exhibited muscule fasciculation, tremor, dyspnea and salivation, that was of lower intensity in animals treated with oxime therapy.

3.1. Dose-response model selection

Supp. Figure 1A shows dose-response model 5 from both model families (exponential and Hill) as the best estimate to AChE activity levels measured at 6 dose levels (including zero dose) for oximes K203 and K027. However, the AIC of the model 5 (AIC_{exp}= -62.44, AIC_{Hill}= -61.7) was more than two units larger than that of the full model (AIC_{full}= -69.84) indicating inappropriate fit. The reason was that the response in the top dose group for oxime K027 deviated substantially from the fitted model (the fitted curve did not hit confidence interval for observed geometric mean response). Thus, we have reanalyzed data leaving out the top dose group for oxime K027. Reanalysis yielded a significant improvement in a model fit with fitted models AIC values (AIC_{exp}= -64.64, AIC_{Hill}= -65.14, Supp. Fig.1B) being more than two units lower than full model's AIC (-61.04). According to the fitted exponential model,

parameter *b* was found to differ significantly between two dose-response curves related to oxime K027 and K203 (exponential: *b*-K203=0,001613 i *b*-K027=0,003566). This implies that oximes differ in potency to reactivate AChE. This model was further extended with allowing parameter *c* (maximum response) to be oxime-dependent, but that assumption was abandoned in further analysis because AIC value decrease was not significant and at the same time the 90%-CIs for parameter *c* were rather wide for both oximes (exponential: *c*-K203 2.70-12600, *c*-K027 2.39-3; Hill: *c*-K203 2.89-Inf, *c*-K027 0.00-3.32), which would cause unreliable estimates.

Finally, the process of model selection resulted in Model 5, both from exponential and Hill family of models, with parameter *b* being oxime-dependent, as an accurate description of the obtained datasets. Table 2 summarizes the selected models parameters with 90%-confidence intervals.

For practical reason of easier folowing the results, as well as the absence of a significant difference in obtained exponential and Hill model analysis, the results of the exponential model are presented below in the text, while both families of the models are presented in Supp. Figures and Tables.

Table 2

3.2. Deriving endpoint-specific value for CESs and related CEDs of oximes

Fitting the four-parameter model to *in vivo* data obtained in this study directly resulted in an estimate of maximum response (*M*) and the within-group standard deviation (*s*) for a reactivation of AChE by oximes, as given in Supp. Fig. 1B and Table 2. In PROAST notation, *M* is equivalent to parameter *c* (for *c*>1, i.e. in increasing curves) and it is expressed as a maximum fold change in response relative to the background (response at dose zero), while *s*

is equvivalent to square root (sqrt) of *var*, which presents within-group variance for the ln-responses. A general theory for defining the effect sizes for continuous endpoints, based on empirical relationship between *M* and *s*, was followed in deriving AChE reactivation-specific values for CES (Slob, 2017). Maximum reactivation of AChE by oximes was estimated to be 2.7-fold, which was translated into a percent change by subtracting one and multiplying with 100 = 170%, while the within-group standard deviation was estimated to be sqrt (0.014) = 0.12. Considering the confidence intervals for both available parameters (*c* and *var*, Table 2) *M* may be between 2.4 and 3, while *s* may be in the range 0.10-0.14. Observed combination of *M* and *s* in our study most closely matches the combination *M* = 2.5 and *s* = 0.131, established by Slob (2017) as quantified empirical relationship between *M* and *s* based on *in vivo* toxicological studies for various endpoints, including AChE activity. Thus, the value of *M* = 2.5 was choosen as a representative maximum fold change of a response for our dataset and associated effect sizes were calculated as follows:

"Large" effect size = $M^{1/2} = 2.5^{1/2} = 1.581$-fold increase or 58%,

"Medium" effect size = $M^{1/4} = 2.5^{1/4} = 1.257$-fold increase or 26%,

"Small" effect size = $M^{1/8} = 2.5^{1/8} = 1.121$-fold increase or 12%.

Calculated critical effect doses (CED) at three levels of effect size were: for oxime K027 $CED_{58} = 52$, $CED_{26} = 24$ and $CED_{12} = 12$ µmol/kg and for oxime K203 $CED_{58} = 100$, $CED_{26} = 46$ and $CED_{12} = 24$ µmol/kg (Supp. Figure 2). Additionaly, Supp. Figure 2 (A3, B3, C3) shows 90%-confidence intervals of estimated CED for both oximes. The same level of effect size (weather it was large, medium or small) was obtained with lower dose of oxime K027 compared to oxime K203 implying higher potency of oxime K027. This was quantitatively examined when additional exponential model, which includes calculation of relative potency factor (RPF) as a model parameter, was fitted to dose-response data combined (Supp. Figure 3). As Supp. Figure 3 shows, oxime K027 was shown to have 1.929 (CI = 1.51, 2.45)-fold

higher reactivating potency (lower CED) than oxime K203 (CED_{K203} = RPF×CED_{K027}) considering DDVP-inhibited AChE. RPF value was found to be independent from the value of CES (constant RPF value was obtained at CES = 12, 26 or 58%), thus being the measure of comparison of whole dose-response curves among treatment groups. However, as the results in Supp. Figure 2 (A3, B3, C3) indicate, scaling the CED confidence intervals was changed with the change in effect size. At small effect size level CED-confidence intervals related to oxime K027 and K203 largely overlapped implying that potency difference could not be distinguished (Supp. Figure 2-C3). At medium effect size level CEDU of oxime K027 overlapped CEDL of oxime K203 implying that potency difference could be very small (Supp. Figure 2-B3). Finally, at the level of large effect size they did not overlap and this results in higher potency rank of oxime K027 in contrast to K203 (Supp. Figure 2-A3). Furthermore, precision in CED estimate, expressed as ratio CEDU/CEDL, increased with increase in effect size (Table 3) resulting in factor 1.6 and 1.7 for oxime K027 and K203, respectively, showing that the information in dose-response data examined was fairly good.

Table 3

4. Discussion

Fitting the dose-response model to datasets obtained in this study resulted in increasing erythrocyte AChE activity trend as a function of dose for both experimental oximes, K027 and K203, in DDVP-exposed rats. However, for oxime K027 at the top dose of 50% LD_{50} significant decline of AChE activity was observed, that considerably influenced the fitted model. Here, it is very important to bear in mind that the fitted models in dose-response modeling do not reflect any biological mechanism, as well as different biological mechanisms may play a role at higher doses as compared to lower doses of compounds (Slob, 2002; Slob

et al., 2005). In this regard, at higher doses intrinsic affinity of an oxime towards intact AChE, which is identified as one of the limiting factor for the maximum therapeutic concentration, may be expected (Voicu et al., 2013; Worek et al., 2016). Further, during complex AChE reactivation process, phosphylated-oxime can be formed, which depending on its stability, can directly re-inhibit reactivated AChE (Luo et al., 1999; Milatović and Jokanović, 2009; Radchenko et al., 2008; Worek et al., 2007). Moreover, it has been shown that dimethylphosphoryl-oxime, expected to be formed by DDVP used in our study, are more potent re-inhibitors of AChE compared to diethylphosphoryl-oximes (Kiderlen et al., 2000; Worek et al., 2000). Furthermore, (Pejchal et al., 2008) have found impaired hepatic excretory function in rats after i.m. administration of K027 at a dose of 50% LD_{50}, which may contribute to its hepatotoxicity, effect that is identified as one of the toxicities of oximes (Balali-Mood et al., 2006; Balali-Mood and Shariat, 1998; Marrs and Vale, 2006). Following on dose-response modeling, aforementioned methodological and biological arguments have led us leaving out the top dose group of oxime K027 in further analysis, because we had assumed that profound decrease in AChE activity was due to toxicity phenomenon caused by oxime itself.

The purpose of dose-response modeling is deriving the dose (critical effect dose, CED) associated with predefined, biologicaly meaningfull size in response/effect (critical effect size, CES). However, the main practical difficulty that arised in our analysis, during modeling AChE activity as a function of oxime dose, was defining the value of CES, that should quantify the level of AChE activity, biologically significant from an antidotal treatment perspective. BMD analysis has been already used for AChE activity as an endpoint, but as a result of inhibition process by OPs and in terms of human health risk assessment, where 20% is accepted as an effect size with no major health consequences for AChE activity in serum (EFSA, 2011). In our experiment AChE activity is perceived as a consequence of reactivation

process by oximes after inhibition by OP-model compound. Thus, CES presents a fold-change or %-change in AChE activity relative to its activity at zero oxime dose, or more specifficaly, relative to the AChE activity in solely DDVP-treated experimental group. This is the main difference in expression of the effect size from the traditional practice used in oxime *in vivo* evaluations, where AChE activity is expressed as % relative to the untreated control (neither OP nor oxime administered). Traditionally expressed, biologicaly meaningful levels of AChE activity in different tissues have been reported so far: erythrocyte AChE activity above 30% of normal level was associated with normal muscule function in pesticide OP-poisoned patients (Thiermann et al., 2005); *in vitro* mouse hemidiaphram AChE activity at 30-40% from the normal level restore muscle function in presence of paraoxon (Thiermann et al., 2010); the minimal level of AChE activity in the pontomedullar area necessary for the survival of nerve agent-intoxicated mice was assessed to be about 5-20% (Bajgar, 1991; Bajgar et al., 1975, 1972, 1971). However, according to our best knowledge, available literature does not allow seting true limits for a minimum extent of AChE reactivation in different tissues granting the survival of an intoxicated organism.

Recently, (Slob, 2017) has established practicly applicable theory for determination of meaningful CESs for toxicological endpoints. Basis of the theory is the fact that the way of expressing the treatment impact is different among different biological endpoints with maximum response (*M*, maximum fold change in response relative to background) and within□group standard deviation (*s*, i.e. variability in response measurements obtained between animals in a dose□group) being the characteristics of an endpoint (Slob and Setzer, 2014). As a result of dose□response analysis of *in vivo* datasets for 27 different endpoints, including AChE activity, quantitative relationship between *M* and *s* was defined, with method for calculation of CESs at three levels of effect size termed large, medium and small (for details see results part and Slob 2017). Applying aforementioned statistical approach to the

maximum response of 2.7-fold and variance of 0.014, obtained for oximes K027 and K203 in our study, yielded AChE activity-specific CESs of 58, 26 and 12% (large, medium and small, respectively).

Setting the CES in dose-response modeling generates associated CED, being influenced by the complete dose-response curve. However, essential output from a dose-response analysis is CED confidence interval, which represents the range in which the true CED lies. It has been recently shown that comparisons of CED confidence intervals, as opposed to direct comparison of single metric (i.e. CED, CEDL or CEDU), comprises a robust way to quantify differences in genotoxic potencies of compounds (Bemis et al., 2016; Soeteman-Hernández et al., 2015; Wills et al., 2017, 2016a, 2016b). Considering CED confidence intervals for the purpose of comparison between oximes K027 and K203 in reactivating eritrocyte AChE in our study revealed that dose-response relationships and potency estimates (e.g. CEDs) are significantly different at 58% effect size where their CED confidence intervals did not overlap resulting in statistically supportable 1.929-fold higher potency/lower therapeutic dose of oxime K027. Identifying therapeutic doses (CED_{58}) of oximes has allowed calculation of therapeutic indexes in terms of evaluation efficacy in relation to their toxicity (median lethal doses, LD_{50}, Antonijevic et al., 2016) as follows:

Therapeutic index, $TI = LD_{50}/CED_{58}$

TI_{K027} = 2528 µmol/kg /52 µmol/kg = 49 vs. TI_{K203} = 716 µmol/kg /100 µmol/kg = 7.

Broader therapeutic index of oxime K027 along with its lower efficacious dose makes it more beneficial compared to oxime K203 in respect of therapeutic dosage regimes of oximes, that include two clinical approaches: repeated intermittent administration or continuous infusion following loading dose (Eddleston and Chowdhury, 2016).

In conclusion, in this study we have utilized the comperhensive combined BMD methodology for the purpose of analysing and quantitative comparing *in vivo* dose-response

data on AChE activity against dose of two experimental oximes, promising reactivators of OP-inhibited AChE. We have identified that dose-response relationship for AChE activity as an endpoint in oximes activity to reactivate OP-inhibited enzyme, could be adequately described by 4-parameter exponential and Hill models, suggesting the new endpoint for BMD analysis. More specifically, we have determined dose-response relationships for experimental oximes K027 and K203 considering reactivation of DDVP-inhibited AChE, described by model m5-*b* ($y=a[c-(c-1)\exp(-bx^d)]$) with parameters a = 0.01453, b-K027 = 0,003566, b-K203 = 0,001613, c = 2.692 and d = 1.208 (i.e. exponential model). Further, oxime K027 was shown to be 1.929-fold more potent oxime compared to oxime K203 considering that 58% increase in AChE activity was achived with dose CED_{58}-K027 = 52 µmol/kg (confidence interval 40, 66 µmol/kg) in contrast to CED_{58}-K203 = 100 µmol/kg (confidence interval 76, 129 µmol/kg). However, at effect sizes up to 26% it could be assumed that reactivating potencies of oximes K027 and K203 might be the same (overlapping CED confidence intervals). Thus, we have cofirmed that comparison of CED confidence intervals, in contrast to a single CED value, presents robust approach to more apparently rank the potenties of compounds.

Finally, we have made efforts to define CES for oximes K027 and K203 for the purpose of their efficacies comparison in our experimental dataset. Defining biologicaly meaningful effect size along with the combined BMD covariate method, would improve the value of experimental data on AChE activity versus oxime dose and provide robust, comparable estimates and interpretations of oximes efficacies.

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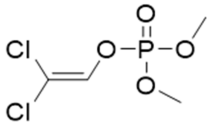
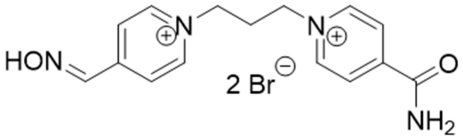
591 **Fig. 1.** Schematic illustration of the benchmark dose (BMD) approach, using hypothetical
592 continous data. In accordance with PROAST software (v65.5, RIVM, The Netherlands)
593 notation equivalent term - critical effect dose (CED) is used for continuous data and it has
594 been applied in this representation. Both response and dose are plotted on the log10-scales.
595 The triangles represent observed geometric mean responses, along with their 90%-confidence
596 intervals, at different tested doses. The solid curve is the best fitted dose–response model.
597 CED is determined from a fitted model as a dose that elicits a pre-specified change in
598 response, denoted critical effect size (CES or equivalent benchmark response, BMR). CES in
599 continuous data is defined as a percent change in mean response relative to the background.
600 The dashed curves represent, respectively, the upper and lower 90% confidence bounds (two-
601 sided) for the effect size as a function of dose. Their intersections with the horizontal line are
602 at the lower and upper bounds of the CED, denoted CEDL and CEDU, respectively. Thus the
603 CEDU-to-CEDL ratio represents the CED estimate precision for a single dataset.

Table 1

Chemical structures, names, molecular weights, tested doses and routes of administration of OP insecticide dichlorvos and oximes in male *Wistar* rats.

Chemical structure	Chemical name	Molecular weight (g/mol)	Dose (µmol/kg)	Route
	dichlorvos (DDVP) *O,O*-dimethyl-*O*-2,2-dichlorovinylphosphate	220.98	30	*s.c.*
	oxime K027 4-carbamoyl-1-(3-{4-hydroxyiminomethylpyridinium-1-yl}propyl)pyridinium dibromide	446.14	0/31/63/126/632/1264	*i.m.*
	oxime K203 4-carbamoyl-1-[(2*E*)-4-{4-hydroxyiminomethylpyridinium-1-yl}but-2-en-1-yl]pyridinium dibromide	458.15	0/9/17/35/179/358	*i.m.*

s.c-subcutaneously, *i.m*-intramuscularly

Table 2

Parameters of exponential and Hill model m5-*b*, with 90%-confidence intervals (CI), that describe reactivation of DDVP-inhibited AChE as a function of oxime K027 and K203 dose in rat erythrocytes.

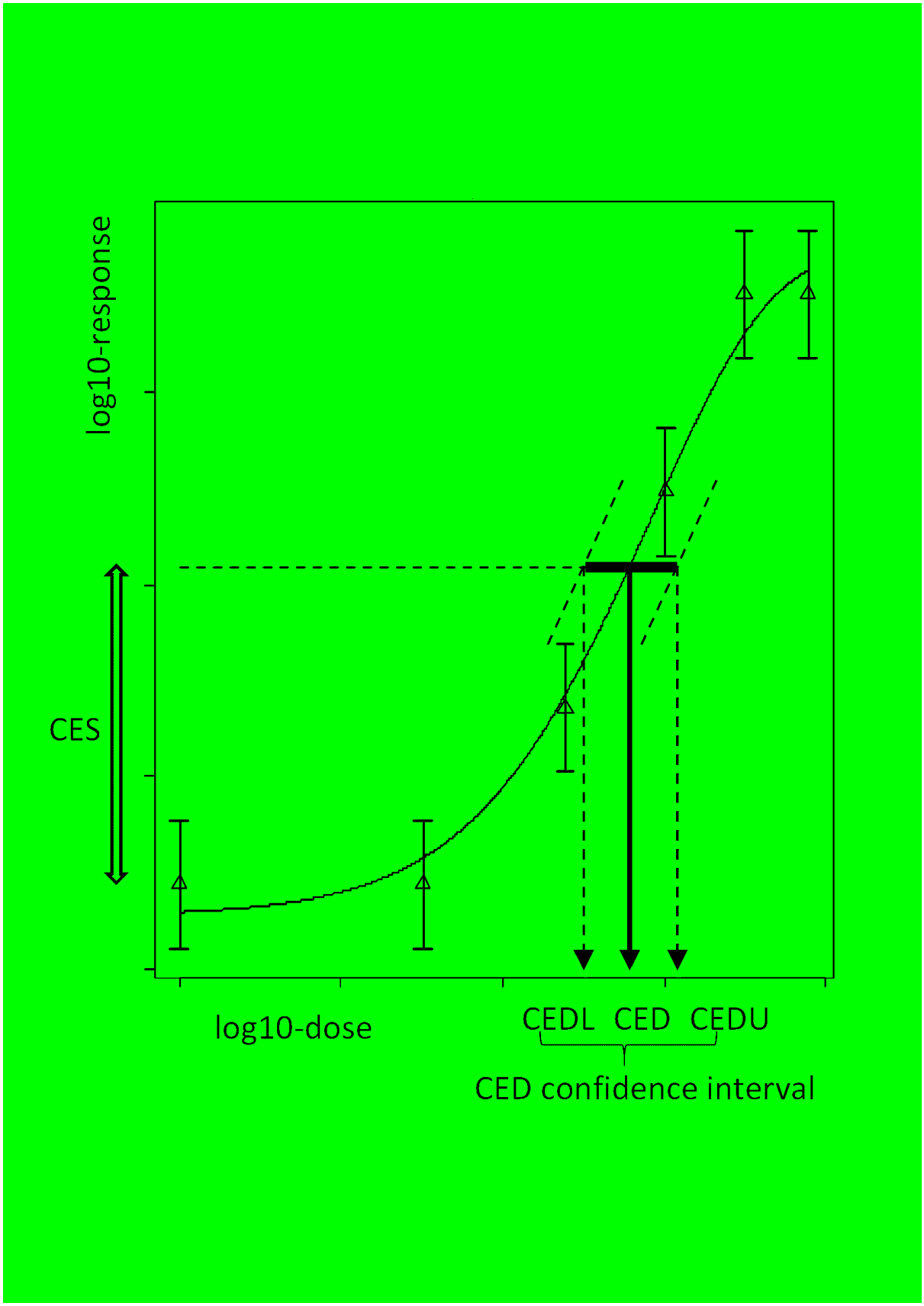
	Exponential m5-b $y = a\,[c - (c\text{-}1)\exp(-bx^d)]$		Hill m5-b $y = a\,[1 + (c\text{-}1)x^d/(b^d+x^d)]$	
parameter	value	90%-CI	value	90%-CI
AIC	-64.64	-	-65.14	-
var-	0.0143	0.0108-0.0202	0.0144	0.0107-0.0201
a-	0.7916	0.7500-0.8340	0.7966	0.7550-0.8380
b-K027	0.003566	0.00107-0.00903	80.85	43.6-69.7
b-K203	0.001613	0.00038-0.00476	154.5	80.5-137
c-	2.692	2.43-2.99	2.77	2.47-3.19
d-	1.208	0.99-1.50	1.503	1.17-1.95

AIC-Akaike information criterion; *var*-variance, *a*-background response, *b*-potency, *c*-maximum response, *d*-steepness.

Table 3

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 erythrocytes.

		Exponential m5-b		Hill m5-b	
		K027	K203	K027	K203
CES=58%	CED	51.9	100.0	50.1	95.8
	CEDL	40.2	75.7	39.2	72.4
	CEDU	65.7	129.0	63.2	124.0
	CEDU/CEDL	1.6	1.7	1.6	1.7
CES=26%	CED	24.2	46.6	25.1	48.0
	CEDL	16.8	31.8	18.0	33.2
	CEDU	33.1	65.7	33.5	67.4
	CEDU/CEDL	2.0	2.1	1.9	2.0
CES=12%	CED	12.3	23.7	14.1	27.0
	CEDL	7.5	14.3	9.0	16.6
	CEDU	18.6	37.1	20.7	41.9
	CEDU/CEDL	2.5	2.6	2.3	2.5


log10-response
CES
log10-dose
CEDL CED CEDU
CED confidence interval

Oximes K027 and K203 reactivate DDVP-inhibited AChE in rat erythrocytes in dose-dependent manner.

4-parameter exponential and Hill models describe dose-dependence of AChE reactivation by oximes K027 and K203.

Maximum size of reactivation of DDVP-inhibited AChE by oximes K027 and K203 was 2.7-fold.

More potent dose-response curve belongs to oxime K027 with relative potency factor of 1.929.