

In vitro evaluation of the catalytic activity of paraoxonases and phosphotriesterases predicts the enzyme circulatory levels required for *in vivo* protection against organophosphate intoxications



Yacov Ashani^a, Haim Leader^b, Nidhi Aggarwal^a, Israel Silman^c, Franz Worek^d,
Joel L. Sussman^b, Moshe Goldsmith^{e,*}

^a Dept. of Structural Biology, The Weizmann Institute of Science, Rehovot, Israel

^b Dept. of Materials and Interfaces, The Weizmann Institute of Science, Rehovot, Israel

^c Dept. of Neurobiology, The Weizmann Institute of Science, Rehovot, Israel

^d The Bundeswehr Institute of Pharmacology and Toxicology, Munich, Germany

^e Dept. of Biological Chemistry, The Weizmann Institute of Science, Rehovot, Israel

ARTICLE INFO

Article history:

Received 18 January 2016

Received in revised form

19 April 2016

Accepted 28 April 2016

Available online 6 May 2016

Keywords:

Catalytic scavengers
Organophosphates
Nerve agent protection
Acetylcholinesterase
Paraoxonase
Phosphotriesterase

ABSTRACT

Catalytic scavengers of organophosphates (OPs) are considered very promising antidote candidates for preventing the adverse effects of OP intoxication as stand alone treatments. This study aimed at correlating the *in-vivo* catalytic efficiency ($(k_{cat}/K_M)[Enzyme]_{pl}$), established prior to the OP challenge, with the severity of symptoms and survival rates of intoxicated animals. The major objective was to apply a theoretical approach to estimate a lower limit for $(k_{cat}/K_M)[Enzyme]_{pl}$ that will be adequate for establishing the desired k_{cat}/K_M value and plasma concentration of efficacious catalytic bioscavengers. Published data sets by our group and others, from *in vivo* protection experiments executed in the absence of any supportive medicine, were analyzed. The k_{cat}/K_M values of eight OP hydrolyzing enzymes and their plasma concentrations in four species exposed to OPs via s.c., i.m. and oral gavage, were analyzed. Our results show that regardless of the OP type and the animal species employed, sign-free animals were observed following bioscavenger treatment provided the theoretically estimated time period required to detoxify 96% of the OP ($t_{96\%}$) *in vivo* was ≤ 10 s. This, for example, can be achieved by an enzyme with $k_{cat}/K_M = 5 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ and a plasma concentration of $0.4 \mu\text{M}$ ($(k_{cat}/K_M)[Enzyme]_{pl} = 20 \text{ min}^{-1}$). Experiments in which animals were intoxicated by i.v. OP injections did not always conform to this rule, and in some cases resulted in high mortality rates. We suggest that *in vivo* evaluation of catalytic scavengers should avoid the unrealistic bolus i.v. route of OP exposure.

© 2016 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Prophylaxis and treatment during the acute phase of intoxication by organophosphate (OP)-based chemical warfare nerve agents (CWNAs) and commercial pesticides is based on antidotal

regimens that consist of pretreatment with pyridostigmine, and post-exposure therapy using atropine, an oxime reactivator, and an anticonvulsant, such as diazepam [1,2]. However, it is well recognized that these drug regimens suffer from several disadvantages that call for new therapeutic strategies [3,4]. One such approach is to rapidly detoxify toxic OPs in the blood before they are able to reach their physiological targets. This objective can be achieved by use of a stoichiometric or a catalytic bioscavenger [5,6]. Use of the best stoichiometric bioscavenger currently available (human butyrylcholinesterase, hBChE) requires administration of hundreds of milligrams of protein to confer protection against toxic doses of a CWNA [7]. Thus, attention has turned to the development of catalytic scavengers, such as recombinant mammalian paraoxonases (PON1s) [8,9] and bacterial phosphotriesterases (PTEs) [10–12],

Abbreviations: OP, organophosphate; AChE, acetylcholinesterase; rePON1, recombinant paraoxonase; PTE, phosphotriesterase; CWNA, chemical warfare nerve agent; GF, cyclosarin; DEFP, O,O-diethyl fluorophosphate.

* Corresponding author.

E-mail addresses: yaacov.ashani@weizmann.ac.il (Y. Ashani), haimleader@gmail.com (H. Leader), nidhi.aggarwal@weizmann.ac.il (N. Aggarwal), israel.silman@weizmann.ac.il (I. Silman), franzworek@bundeswehr.org (F. Worek), joel.sussman@weizmann.ac.il (J.L. Sussman), moshe.goldsmith@weizmann.ac.il (M. Goldsmith).

with the objective of evolving enzymes capable of effective degradation of a broad spectrum of OP nerve agents. The ultimate goal is to provide protection against OPs at protein doses an order of magnitude lower than the hundreds of milligram doses required of human BChE.

The initial criterion for success in screening mutant libraries for catalytic activity was set at $k_{\text{cat}}/K_M \geq 1 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ when reacting with OPs. Progress made already suggests that this value is indeed achievable [8,9,11,12]. Protection experiments *in vivo* confirmed the capacity of catalytic scavengers such as wt parathion hydrolase from *Pseudomonas* sp. [13,14], mammalian PONs [15,16], PTEs from *Brevudimonas diminuta* [17], and OpdA from *Agrobacterium radiobacter* [18–20], to prevent lethality at less than 1 mg/kg of catalytic enzyme, and even to confer sign-free protection in several cases in animal intoxication models. It should be pointed out that although evolved variants of bacterial OP hydrolases display high catalytic activity against a variety of nerve agents [8–12], their wt forms as well, as well as mammalian wt OP hydrolases, are considered to be poor antidotes for treatment of nerve agent intoxication [6].

The goal of this report was to set a lower quantitative boundary for the *in vivo* product of k_{cat}/K_M and the plasma concentration of the catalytic enzyme, $(k_{\text{cat}}/K_M)[\text{Enzyme}]_{\text{pl}}$, based on published *in vivo* protection experiments. This value should predict the amount and catalytic characteristics that would suffice to confer near sign-free protection against intoxication by a given OP as a stand alone, prophylactic treatment. Establishment of such values can guide future decisions regarding the development of catalytic scavengers. We analyzed data from experiments in which a variety of OP hydrolases were used alone (*viz.*, in the absence of oximes/atropine/diazepam) to protect against lethal OP doses, and propose desirable k_{cat}/K_M values to comply with the requirement for an effective low dose of a catalytic scavenger antidote. It is further proposed that the model can be reliably extrapolated to predict efficacy of catalytic scavengers in humans.

2. Materials and methods

2.1. Materials

The wt-like-G3C9-derived PON1 variants 2D8, IIG1, VIID2 and PG11 were expressed and purified as described by Goldsmith et al. [9]. Variant 11C1 was generated by M. Goldsmith et al. (2012). The evolution of rePON1 4E9 was reported by Gupta et al. [8], and PTE-C23 was described by Cherny et al. [11]. Human acetylcholinesterase (rhAChE) was expressed and purified in the Israeli Center for Structural Proteomics (ISPC), at the Weizmann Institute of Science. Cyclosarin (GF) was obtained *in situ*, in water, at non-hazardous concentrations, as described earlier [8].

2.2. *In vitro* protection

The *in vitro* protection of rhAChE against GF inhibition by several rePON1s (2D8, IIG1, VIID2 and PG11) was performed in 0.1% Tergitol/1 mM CaCl_2 /50 mM NaCl/50 mM Tris, pH 8.0, at 25 °C. Mixtures of 3.5–14.0 nM rhAChE and 0.6–8.6 μM rePON1 were spiked with 0.13–0.60 μM GF to initiate the protection study. The decrease in rhAChE baseline activity (AChE_0 ; OD/min at 412 nm), was monitored according to Ellman et al. [21] employing 0.5 mM ATC and 0.4 mM DTNB in 50 mM phosphate, pH 8.0, until no further change in activity was observed. This activity is dubbed AChE_∞ .

2.3. *In vivo* protection

Most of the data used to establish the lower boundary for the

value of $(k_{\text{cat}}/K_M)[\text{Enzyme}]_{\text{pl}}$ were taken from published data, exception for the data relating to the effect of the 11C1 variant on intoxication of rats by paraoxon and parathion (Goldsmith et al. 2012). The data collated relate to experiments that tested the protection of mice, rats, guinea pigs and monkeys against intoxication by the nerve agents GF, VX, tabun (GA), and the pesticides paraoxon, parathion and dichlorvos. Only experiments in which the catalytic enzymes were used alone (*viz.*, in the absence of oximes/atropine/diazepam) were considered. The studies considered were limited to those in which the levels of the enzyme in the plasma were directly determined prior to OP exposure, or inferred from independent pharmacokinetic studies of the same enzyme in the same species. For monkeys protected against dichlorvos by OpdA, and rats against methyl parathion the plasma enzyme level was estimated as described under Results (4.3). k_{cat}/K_M values were usually determined and reported at 25 °C, and assumed to be 2-fold greater at 37 or 38 °C. In some cases they were measured at 37 °C and 38 °C.

3. Theory and calculations

Calculation of the time required to hydrolyze 96% of the OP substrate in an animal's plasma was based on the classical Michaelis-Menten equation:

$$v = \frac{V_{\text{max}}[\text{OP}]}{K_M + [\text{OP}]} \quad (1)$$

that was simplified by assuming that the [OP] concentration in the plasma that produces toxicity following an i.v. dose of $2 \times \text{LD}_{50}$ is in the micromolar range, and that the corresponding K_M is at least ≥ 10 -fold greater. For example, an i.v. LD_{50} dose of GA, GB or VX in monkeys [22] can theoretically produce maximal plasma concentrations of 6, 2, and 0.6 μM OP, respectively, while the K_M values for GA, GB, and VX were reported to be 101, 700 and 434 μM , respectively [14,23,24], and those for the pesticides parathion and dichlorvos were reported to be 110 and 183 μM , respectively [18]. When $K_M \gg [\text{OP}]$, the Michaelis-Menten velocity term, v , is approximated by $v = V_{\text{max}}[\text{OP}]/K_M$. Since $V_{\text{max}} = k_{\text{cat}}[\text{Enzyme}]_{\text{pl}}$, the velocity equation in plasma is reduced to a first-order equation:

$$v = (k_{\text{cat}}/K_M)[\text{Enzyme}]_{\text{pl}}[\text{OP}] \quad (2)$$

where $[\text{Enzyme}]_{\text{pl}}$ is the molar concentration of the catalytic enzyme in plasma, and the product $(k_{\text{cat}}/K_M)[\text{Enzyme}]_{\text{pl}}$ is the pseudo-first-order rate constant (k_{obs}) for hydrolysis of the OP. The time required to detoxify 96% of the OP was obtained by calculating the value of 5xhalf-lives in accordance with Eq. (3):

$$t_{96\%} = 5 \times 0.69/k_{\text{obs}} = 3.45 / \left\{ (k_{\text{cat}}/K_M)[\text{Enzyme}]_{\text{pl}} \right\} \quad (3)$$

For k_{cat}/K_M given in $\text{M}^{-1} \text{ min}^{-1}$ $t_{96\%}$ is expressed in minutes. When the OP concentration in the plasma was expected to be in the range of the value of K_M , the integrated form of the Michaelis-Menten equation was used to calculate the time required to reduce the OP concentration to 4% of its presumed baseline plasma concentration [25].

The graph that demonstrates the *in vitro* correlation between k_{cat}/K_M , the catalytic scavenger concentration, and the protection of AChE is based on a modified version of an equation published earlier [26]:

$$-\ln \frac{[\text{AChE}]_\infty}{[\text{AChE}]_0} = \frac{k_i[\text{OP}]_0}{(k_{\text{cat}}/K_M)[\text{E}]_0} \quad (4)$$

where $[\text{OP}]_0$ and $[\text{E}]_0$ are the molar concentrations of the OP and the catalytic scavenger, respectively, and k_i and k_{cat}/K_M are,

respectively, the second-order rate constant for the inhibition of AChE by the OP, and the rate constant for the detoxification of the OP by the catalytic scavenger, E, wherein $K_M \gg [OP]$. Here, when an OP is added to a mixture of AChE and a catalytic scavenger, at infinite time, when all the OP has either been hydrolyzed or sequestered by AChE, the residual activity of the AChE is given by Eq. (4).

4. Results

4.1. Validation of Eq. (4) in buffer solutions

Fig. 1 depicts the validation of Eq. (4), showing the plot of the observed vs. the calculated residual AChE activity at $t = \infty$. At infinite time, the GF spiked into a rhAChE/rePON1 reaction mixture was completely sequestered by two pathways: stoichiometric reaction with AChE, and parallel detoxification by the rePON1. The straight line with slope = 1 suggests a linear 1:1 correlation among the involved kinetic constants, the concentrations of the OP and the protecting enzyme. Thus, knowledge of the k_{cat}/K_M of a given rePON1 (and presumably of any OP hydrolase) and of the second-order rate constant for the inhibition of AChE by GF (k_i) permits accurate prediction of the level of protection of AChE provided by the catalytic scavenger.

4.2. Protection against an i.v. challenge

In all listed cases where OPs were challenged via the i.v. route (Table 1), 3.0–7.3 protective ratios were obtained at 24 h by pre-treatment alone with relatively low doses of catalytic bioscavengers (<1 mg/kg). The biological end-point in these experiments was the survival ratio. Non-treated animals did not survive any of the reported challenges. Yet, despite the success achieved in reducing in mortality, most surviving animals suffered from mild to moderate cholinergic symptoms that diminished visibly after several hours. The expected high OP concentrations in the plasma at $t = 0$ were estimated to be near the K_M values (see references listed in Table 1). Thus, the theoretical time to detoxify 96% of the injected OP was calculated for the i.v. exposures by applying the integrated form of the Michaelis-Menten equation [25]. To this end we assumed a

rapid and homogeneous encounter between the challenge and the scavenger within the plasma *in vivo*.

4.3. Protection against OPs administered via s.c., i.m. and oral gavage routes

Table 2 summarizes the second group of published data in which the toxicological endpoints for the enzyme-treated animals experiment were recorded as cholinergic signs, i.e., no death reported. In these studies, with one exception (one out of 11 animals in the paraoxon-11C1 study), no deaths were observed. It seems that in order to achieve sign-free animals the theoretical time to detoxify 96% of the OP needs to be < 10 s. Increasing $t_{96\%}$ increases the number and the severity of visible intoxication symptoms. For all cases classified under sign-free animals, in all likelihood the relatively slow entry of the OP into the blood stream permitted the circulating scavenger to detoxify it effectively, thus preventing its penetration into physiologically important sites in significant amounts.

Of special importance are the results reported by Jackson et al. [20], wherein the catalytic scavenger, a wt OpdA, was demonstrated for the first time to confer full and sign-free protection in a non-human primate. Thus, as low as 1.2 mg/kg enzyme alone conferred remarkable protection against 3xLD₅₀ (75 mg/kg) of orally administered dichlorvos. Gresham et al. [19] reported sign-free animals following injection of as little as 0.15 mg/kg of the same wt OpdA to rats challenged with 3xLD₅₀ methyl parathion. Unfortunately, the published PK data for wt OpdA in the same species [19,27] could not be translated into molar concentrations. It was therefore assumed that half of the enzyme dose administered was available for detoxification following i.v. injection of the OpdA, and the maximal enzyme level was thus expected to be 0.05 and 0.43 μM in rats and monkeys, respectively. In monkeys, the theoretical time to detoxify 96% dichlorvos 1 min after its oral load was calculated to be 7.5 s, and it seems that despite the relatively rapid decline in the OpdA levels in the monkeys' plasma ($t_{1/2}$, 40 min; [27]) its k_{cat}/K_M enabled efficient detoxification to such an extent that no apnea, or heart and respiratory rate abnormalities were observed over 250 min. Non-protected monkeys developed severe toxic signs within 10–20 min. In the case of rats protected with OpdA against methyl parathion, the extremely low circulating OpdA concentration (0.05 μM) was compensated for by the high k_{cat}/K_M ($72 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$) and no adverse effects were observed in the first 16 h post oral exposure to 3xLD₅₀ (30 mg/kg) of methyl parathion. Within the non-protected group 7/8 died within 159 min.

5. Discussion

The main objective of this study was to set a lower boundary for the *in vivo* dose of a catalytic bioscavenger, based on published data, which will suffice, when employed alone, to confer reasonable protection against OP intoxication. Catalytic bioscavengers can offer protection at protein doses several fold lower than those required for stoichiometric antidotes. Furthermore, the dose required can be established on the basis of the value of k_{cat}/K_M , which is readily obtained by *in vitro* evaluation of the kinetics of detoxification of the relevant OP. The *in vitro* validation of Eq. (4) (Fig. 1), together with the previously published *ex vivo* demonstration of protection of ChEs in human whole blood by selected catalytic scavengers [26], clearly show that the combined knowledge of k_{cat}/K_M and of the molar concentration of the detoxifying enzyme not only forecasts protection capacity, but also accurately predicts the level of protection of blood ChEs, at least *in vitro*. The question then arose as to how this 'proof-of-concept' could be tested *in vivo*, so as to establish

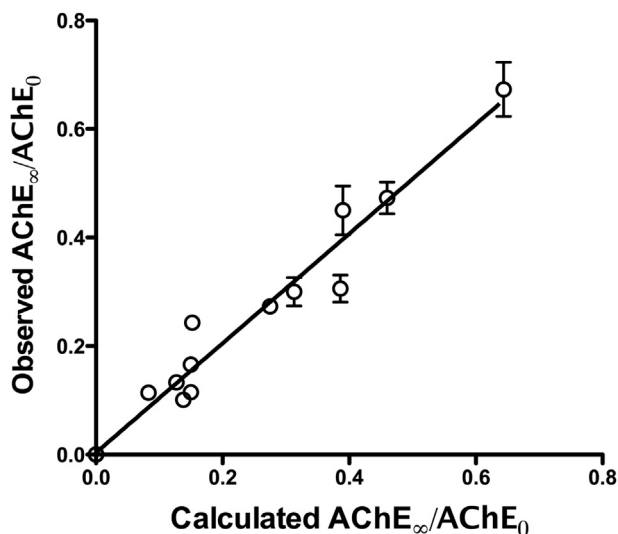


Fig. 1. Validation of Eq. (4) for a variety of rePON1s protecting hAChE against GF. The slope of the straight line is 1.0. The line was constructed for rePON1s: wt G3C9, 2D8, 4E9, PG11, 1-F11 and VIIIID2. Bars denote SD ($n = 2-3$). (see Materials and methods for experimental conditions).

Table 1

Protective ratio in mice pretreated i.v. with catalytic scavengers and challenged i.v. with tabun, paraoxon, DEFP and the cyclosarin surrogate, Sp-CMP.

OP	Enzyme (mg/kg)	$k_{cat}/K_M \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$	Enzyme plasma conc ^c (μM) ^a	Protective ratio (at 24 h) ^b	$t_{96\%}$ seconds ^c (38 °C)	Reference
Tabun, GA	Parathion hydrolase(0.36)	4.6 (25 °C)	0.25	3.9 (n = 16)	8	[14]
Tabun, GA	Parathion hydrolase(0.53)	4.6 (25 °C)	0.37 ^d	5.7 (n = 16)	5.5	[14]
Paraoxon	Parathion hydrolase(0.18)	140 (38 °C)	0.12 ^d	3.8 (n = 16)	1.1	[13]
Paraoxon	Parathion hydrolase(0.36)	140 (38 °C)	0.25	7.3 (n = 16)	0.53	[13]
DEFP ^e	Parathion hydrolase(0.62)	5.2 (38 °C)	0.43 ^d	2.9 (n = 16)	8.7	[13]
Sp-CMP ^e	rePON1, 4E9(2.2)	1.7 (25 °C)	0.30 ^f	50% survived LD ₁₀₀ dose (n = 14)	19	[8]

^a Determined by the listed authors prior to the OP challenge and within 5–10 min of enzyme load.^b LD₅₀ in protected mice divided by LD₅₀ in non-protected animals (1xLD₅₀: tabun, 0.22 mg/kg, paraoxon, 0.48 mg/kg, DEFP, 0.65 mg/kg). LD₁₀₀ of Sp-CMP, 0.29 mg/kg.^c Calculated for 38 °C by using the integrated form of Michaelis-Menten equation and the biochemical constants reported in the cited references. The kinetic constants determined at 25 °C were assumed to be 2-fold greater at 38 °C.^d The enzyme plasma concentration estimated from PK determined for different enzyme doses, and assuming approximately linear increase in concentration upon increasing the enzyme dose.^e DEFP, O,O-diethyl fluorophosphate; Sp-CMP, the toxic Sp enantiomer of a GF surrogate in which the fluoride leaving group was replaced by a coumarin derivative.^f Determined at 6 h post enzyme load, 5 min before OP challenge.**Table 2**

Summary of protection experiments in guinea pigs, rats, and monkeys pre- and post-treated i.v. using catalytic scavengers, and challenged via s.c., i.m., and oral gavage with OPs.

OP (injection route)	Enzyme (mg/kg) modality	$k_{cat}/K_M \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$	Enzyme plasma level (μM) ^a	$t_{96\%}$ (sec) 37 °C ^b	Toxicity level ^c	Reference
GF(s.c.)	rePON1-IIG1 (1.0) pre-treatment	3.4 (25 °C) 8.4(37 °C)	0.3	8.2	I	[17]
Paraoxon (i.m)	rePON1-11C1(1.0) post-treatment	2.2 (25 °C)	≈0.5	9.4	I	Goldsmith et al. (2012)
Parathion ^d (oral gavage)	rePON1-11C1(2.0) post-treatment	2.2 (25 °C)	1.1	4.2	I	Goldsmith et al. (2012)
Methyl parathion (oral gavage)	OpdA (0.15) Post-treatment	72.0	<0.05 ^e	1.1	I	[19,27]
Dichlorvos (oral gavage)	OpdA(1.2) post-treatment	6.6 (37 °C)	<0.42 ^e	7.5	I	[20,27]
VX(s.c.)	PTE-C23(5.0) post-treatment	0.5 (25 °C)	1.3	15.9	II	[11,16]
GF(s.c.)	rePON1-IIG1 (0.2) pre-treatment	3.4 (25 °C) 8.4(37 °C)	0.08	30.8	III	[17]
VX(s.c.)	PTE-C23(2.0) post-treatment	0.5 (25 °C)	0.5	41.4	III	[16]

^a Plasma levels prior to exposure reported by the cited authors with the exception of OpdA. Animal employed: For GF and VX, guinea pigs; For paraoxon, parathion, and methyl parathion, rats; For dichlorvos, monkeys.^b Calculated for 37 °C by using eq. (3) and the biochemical constants reported in the cited references. The kinetic constants determined at 25 °C were assumed to be 2-fold greater at 37 °C.^c The severity of the toxic signs was classified as: I, practically sign-free or only local fasciculations at the OP injection site; II, Moderate, transient salivation, and transient breathing difficulties; III, Severe, non-lethal: transient respiratory depression, salivation, convulsions.^d The k_{cat}/K_M used for parathion was that of paraoxon, which is the toxic metabolite of parathion. 11C1 displayed poor reactivity towards parathion.^e See Results, paragraph 5.3 for explanation.

the minimal value of the product of $(k_{cat}/K_M)[\text{Enzyme}]_{pl}$ required to confer full and sign-free protection?

To this end, 96% detoxification in less than one blood circulation time was selected as the desirable criterion. This choice was based on the fact that 0.2 LD₅₀ doses of sarin, soman or VX, administered s.c. in guinea pigs [28], or dichlorvos loaded via oral gavage in rats [29], did not produce adverse effects even when administered chronically on a daily basis. Since this study considered published reports in which doses of up to 7.5xLD₅₀ OP were administered (Table 1), it seemed reasonable to require a combination of catalytic efficiency and enzyme level that would rapidly decrease the level of the OP in the plasma level by ≥ 25 -fold.

Table 1 suggests that, even though the theoretical values of $t_{96\%}$ for paraoxon were reached at 0.5–1 s, onset of toxic signs was not completely prevented. Yet, considering the i.v. LD₅₀ values of tabun, paraoxon, and DEFP administered i.v., and the protective ratios achieved, the small dose of parathion hydrolase displayed excellent efficacy given the fact that no post-challenge supportive medicine was applied. This can be ascribed to the combination of high k_{cat}/K_M values and the available enzyme levels at the time of challenge. It is worth pointing out that for the slowest calculated detoxification rate, it takes 19 s to hydrolyze 96% of Sp-CMP (Table 1), the dose given was sufficient to save 50% of the animals, and it seems that despite the relatively high $t_{96\%}$ (19 s), the low toxic dose, compared to the other OPs, allowed effective removal of the anti-AChE in the

circulation.

As is seen from Table 2, for the non-i.v. routes of OP administration, practically sign-free protection was reported when a theoretical value of 96% detoxification was achieved within <10 s, regardless of the OP used, its route of administration, or the type of animal employed. Thus challenges with paraoxon (rats), parathion (rats), dichlorvos (monkeys and rats), and GF (guinea pigs) all resulted in sign-free animals as long as detoxification was theoretically calculated to reach 96% in less than 10 s due to the favorable value of $k_{cat}/K_M[\text{Enzyme}]_{pl}$.

When data reported for s.c., i.m. and oral gavage challenge (Table 2) were compared to data for i.v. administration of the OPs (Table 1) it became clear that the i.v. route is by far more severe. As far as rates of absorption and body distribution are concerned, it is far from being a realistic scenario. In all likelihood, sign-free animals are not expected to be observed. What is anticipated is that following i.v. bolus administration of relatively large OP doses, a sufficiently large amount will reach the physiologically important AChE sites before the challenge has had the chance to come into full and homogeneous contact with the circulating scavenger. It is, therefore, suggested that for all practical purposes evaluation of catalytic scavengers should be limited to s.c., i.m., oral gavage, inhalation and percutaneous exposures.

The stoichiometric scavenger human butyrylcholinesterase was shown to be very effective against percutaneous (p.c.) exposure to

VX even when administered at 2 h post-challenge (protein dose, 72 mg/kg) [30]. To the best of our knowledge, there are no published studies on the efficacy of catalytic scavengers against p.c. poisoning by V-type nerve agents. Skin penetration is a most realistic intoxication scenario for V agents. Their slow rate of entry increases the chances of effective hydrolysis by the circulating catalytic scavenger, thereby preventing penetration of V agents into physiologically important sites. However, the later onset of toxic signs raises the question of whether the catalytic scavenger will still be present at an adequate concentration to hydrolyze effectively nerve agent that has penetrated slowly. In this respect it is important to note that in general bacterial wt PTEs display short half-life values (~40–50 min) in mice [13], rats [19] and monkeys [27]. However, a modified PTE variant displayed enhanced stability with $t_{0.5} = 170$ min in guinea pigs [16]. It is clear that one of the next challenges in the development of catalytic scavengers is to achieve significant prolongation of their circulatory residence time while retaining efficacy and safety.

6. Conclusions

The fact that similar lower boundaries for the product (k_{cat}/K_M) [Enzyme]_{pl} were defined for sign-free animals, regardless of the OP type, its entry route, or the species employed, offers a rational approach to the decision of whether a particular OP hydrolase may be considered as a promising antidote for use in humans. Assuming that only 60% of an enzyme administered i.m. to a human subject will be present in the circulation in a catalytically active form at the time of challenge, a dose of ~2 mg/kg of a 40 kDa enzyme is expected to provide a concentration of ≥ 0.4 μM enzyme in the plasma. At this plasma concentration, the desired k_{cat}/K_M should be $5 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ to confer protection without symptoms of intoxication. Variants with somewhat lower k_{cat}/K_M values, e.g., $2.5 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ or given at a lower dose (e.g. 1 mg/kg) are likely to confer significant protection (prevent morbidity) when administered as a stand-alone treatment; intoxication symptoms are, however, to be expected. The actual lower limit for the required dose of a given bioscavenger enzyme can, of course, only be accurately estimated after determination of its PK in humans. An OP injected i.v. at a high concentration creates a bolus that is difficult to overcome even with a highly efficient bioscavenger.

Acknowledgements

Financial support by the Defense Threat Reduction Agency HDTRA1-11-C-0026, the US national Institutes of Health 2U54NS058183-06, and the Israel Ministry of Industry/Office of the Chief Scientist Kamin project #50256 are gratefully acknowledged.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.cbi.2016.04.039>.

References

- [1] T.C. Marrs, P. Rice, J.A. Vale, The role oximes in the treatment of nerve agent poisoning in civilian casualties, *Toxicol. Rev.* 25 (2006) 297–323.
- [2] H. Thiermann, F. Worek, K. Kehe, Limitations and challenges in treatment of acute chemical warfare agent poisoning, *Chem-Biol Interact.* 206 (2013) 435–443.
- [3] M.A. Dunn, F.R. Sidell, Progress in medical defense against nerve agents, *JAMA* 262 (5) (1989) 649–652.
- [4] P. Masson, Evolution of and perspectives on therapeutic approaches to nerve agent poisoning, *Toxicol. Lett.* 206 (1) (2011) 5–13.
- [5] B.P. Doctor, A. Saxena, Bioscavengers for the protection of humans against organophosphate toxicity, *Chem-Biol Interact.* 157–158 (2005) 167–171.
- [6] D.E. Lenz, D. Yeung, J.R. Smith, R.E. Sweeney, L.A. Lumley, D.M. Cerasoli, Stoichiometric and catalytic scavengers as protection against nerve agent toxicity: a mini review, *Toxicology* 233 (2007) 313–319.
- [7] Y. Ashani, S. Pistinner, Estimation of the upper limit of human butyrylcholinesterase dose required for protection against organophosphates toxicity: a mathematically based toxicokinetic model, *Toxicol. Sci.* 77 (2004) 358–367.
- [8] R.D. Gupta, M. Goldsmith, Y. Ashani, Y. Simo, Y. Mullochandrov, H. Bar, M. Ben-David, H. Leader, R. Margalit, I. Silman, J.L. Sussman, D.S. Tawfik, Directed evolution of hydrolases for prevention of G-type nerve agent intoxication, *Nat. Chem. Biol.* 7 (2011) 120125.
- [9] M. Goldsmith, Y. Ashani, Y. Simo, M. Ben-David, H. Leader, I. Silman, J.L. Sussman, D.S. Tawfik, Evolved stereoselective hydrolases for broad-spectrum G-type nerve agent detoxification, *Chem. Biol.* 19 (2012) 456–466.
- [10] P.C. Tsai, N. Fox, A.N. Bigley, S.P. Harvey, D.P. Barondeau, F.M. Raushel, Enzymes for the homeland defense: optimizing phosphotriesterase for the hydrolysis of organophosphate nerve agents, *Biochemistry* 51 (2012) 6463–6475.
- [11] I. Cherny, P. Greisen Jr., Y. Ashani, S.D. Khare, G. Oberdorfer, H. Leader, D. Baker, D.S. Tawfik, Engineering V-type nerve agents detoxifying enzymes using computationally focused libraries, *ACS Chem. Biol.* 8 (2013) 2394–2403.
- [12] A.N. Bigley, M.F. Mabanglo, S.P. Harvey, F.M. Raushel, Variants of phosphotriesterase for the enhanced detoxification of the chemical warfare agent VR, *Biochemistry* 54 (2015) 5502–5512.
- [13] Y. Ashani, N. Rothschild, Y. Segall, D. Levanon, L. Raveh, Prophylaxis against organophosphate poisoning by an enzyme hydrolyzing organophosphorus compounds in mice, *Life Sci.* 49 (1991) 367–374.
- [14] L. Raveh, Y. Segall, H. Leader, N. Rothschild, D. Levanon, Y. Ashani, Protection against tabun intoxication in mice by prophylaxis with an enzyme hydrolyzing organophosphate esters, *Biochem. Pharmacol.* 44 (1992) 397–400.
- [15] M. Valiyaveetil, Y. Alamneh, P. Rezk, M.W. Perkins, A.M. Sciuto, B.P. Doctor, M.P. Nambiar, Recombinant paraoxonase 1 protects against sarin and soman toxicity following microinstillation inhalation exposure in guinea pigs, *Toxicol. Lett.* 202 (2011) 203–208.
- [16] F. Worek, T. Seeger, M. Goldsmith, Y. Ashani, H. Leader, J.S. Sussman, D. Tawfik, H. Thiermann, T. Wille, Efficacy of the rePON1 mutant IIG1 to prevent cyclosarin toxicity in vivo and to detoxify structurally different nerve agents in vitro, *Arch. Toxicol.* 88 (6) (2014) 1257–1266.
- [17] F. Worek, T. Seeger, G. Reiter, M. Goldsmith, Y. Ashani, H. Leader, J.L. Sussman, N. Aggarwal, H. Thiermann, D.S. Tawfik, Post-exposure treatment of VX poisoned guinea pigs with the engineered phosphotriesterase mutant C23: a proof-of-concept study, *Toxicol. Lett.* 231 (1) (2014) 45–54.
- [18] S.B. Bird, T.D. Sutherland, C. Gresham, J. Oakeshott, C. Scott, M. Eddleston, OpdA, a bacterial organophosphorus hydrolase, prevents lethality in rats after poisoning with highly toxic organophosphorus pesticides, *Toxicology* 247 (2008) 88–92.
- [19] C. Gresham, C. Rosenbaum, R.J. Gaspari, C.J. Jackson, S.B. Bird, Kinetics and efficacy of an organophosphorus hydrolase in a rodent model of methylparathion poisoning, *Acad. Emerg. Med.* 17 (7) (2010) 736–740.
- [20] C.J. Jackson, A. Carville, J. Ward, K. Mansfield, D.L. Ollis, T. Khurana, S.B. Bird, Use of OpdA, an organophosphorus (OP) hydrolase, prevents lethality in an African green monkey model of acute OP poisoning, *Toxicology* 317 (2014) 1–5.
- [21] G.L. Ellman, K.D. Courtney, V. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, *Biochem. Pharmacol.* 7 (1961) 88–95.
- [22] A.B. Munro, K.R. Ambrose, A.P. Watson, Toxicity of organophosphate chemical warfare agents GA, GB, and VX: implications for public protection, *Environ. Health Perspect.* 102 (1994) 18–28.
- [23] D.P. Dumas, H.D. Durst, W.G. Landis, F.M. Raushel, J.R. Wild, Inactivation of organophosphorus nerve agents by the phosphotriesterase from *Pseudomonas diminuta*, *Arch. Biochem. Biophys.* 277 (1990) 155–159.
- [24] V.K. Rastogi, J.J. De Frank, T.C. Cheng, J.R. Wild, Enzymatic hydrolysis of russian VX by organophosphorus hydrolase, *Biochem. Biophys. Res. Comm.* 241 (1997) 294–296.
- [25] I.H. Segel, *Biochemical Calculations*, first ed., John Wiley & Sons, Inc., New-York, 1968.
- [26] Y. Ashani, M. Goldsmith, H. Leader, I. Silman, J.L. Sussman, D.S. Tawfik, In vitro detoxification of cyclosarin in human blood pre-incubated *ex vivo* with recombinant serum paraoxonases, *Toxicol. Lett.* 206 (2011) 24–28.
- [27] C.J. Jackson, C. Scott, A. Carville, K. Mansfield, D.L. Ollis, S.B. Bird, Pharmacokinetics of OpdA, an organophosphorus hydrolase, in the African green monkey, *Biochem. Pharmacol.* 80 (2010) 1075–1079.
- [28] C.R. Atchison, R.E. Sheridan, S.M. Duniho, T.M. Shih, Development of a guinea pig model for low-dose, long-term exposure to organophosphorus nerve agents, *Toxicol. Mech. Methods* 14 (2004) 183–194.
- [29] J. Yang, X. Sun, Z. Feng, D. Hao, M. Wang, X. Zhao, C. Sun, Metabolomic analysis of the toxic effects of chronic exposure to low-level dichlorvos on rats using ultra-performance liquid chromatography-mass spectrometry, *Toxicol. Lett.* 206 (2011) 306–313.
- [30] H. Mumford, J.K. Troyer, Post exposure therapy with recombinant human BuChE following percutaneous VX challenge in guinea pigs, *Toxicol. Lett.* 206 (2011) 29–34.