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Post-exposure treatment of VX poisoned guinea pigs with the engineered phosphotriesterase mutant C23: A proof-of-concept study

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HIGHLIGHTS

- We investigated the therapeutic effect of the phosphotriesterase mutant C23 *in vivo*.
- Post-exposure C23 therapy prevented lethality and minimized signs in VX poisoned guinea pigs. C23 therapy resulted in rapid elimination of the toxic P(−) VX enantiomer.
- This proof-of-concept study gives insight in the potential of catalytic bioscavengers in nerve agent poisoning.

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ABSTRACT

The highly toxic organophosphorus (OP) nerve agent VX is characterized by a remarkable biological persistence which limits the effectiveness of standard treatment with atropine and oximes. Existing OP hydrolyzing enzymes show low activity against VX and hydrolyze preferentially the less toxic P(+)-VX enantiomer. Recently, a phosphotriesterase (PTE) mutant, C23, was engineered towards the hydrolysis of the toxic P(−) isomers of VX and other V-type agents with relatively high *in vitro* catalytic efficiency ($k_{cat}/K_M = 5 \times 10^6\ M^{-1}\ min^{-1}$). To investigate the suitability of the PTE mutant C23 as a catalytic scavenger, an *in vivo* guinea pig model was established to determine the efficacy of post-exposure treatment with C23 alone against VX intoxication. Injection of C23 ($5\ mg\ kg^{-1}$ i.v.) 5 min after s.c. challenge with VX ($\sim 2LD_{50}$) prevented systemic toxicity. A lower C23 dose ($2\ mg\ kg^{-1}$) reduced systemic toxicity and prevented mortality. Delayed treatment (i.e., 15 min post VX) with $5\ mg\ kg^{-1}$ C23 resulted in survival of all animals and only in moderate systemic toxicity. Although C23 did not prevent inhibition of erythrocyte acetylcholinesterase (AChE) activity, it partially preserved brain AChE activity. C23 therapy resulted in a rapid decrease of racemic VX blood concentration which was mainly due to the rate of degradation of the toxic P(−)-VX enantiomer that correlates with the C23 blood levels and its k_{cat}/K_M value. Although performed under anesthesia, this proof-of-concept study demonstrated for the first time the ability of a catalytic bioscavenger to prevent systemic VX toxicity when given alone as a single post-exposure treatment, and enables an initial assessment of a time window for this approach. In conclusion, the PTE mutant C23 may be considered as a promising starting point for the development of highly effective catalytic bioscavengers for post-exposure treatment of V-agents intoxication.

1. Introduction

The recent use of the highly toxic organophosphorus (OP) nerve agent sarin in Syria emphasizes the need to develop effective medical countermeasures against nerve agent intoxications (Eisenkraft et al., 2014). The high toxicity of OP nerve agents ensues from the irreversible inhibition of the pivotal enzyme acetylcholinesterase

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(AChE) resulting in overstimulation of peripheral and central cholinergic receptors (Marrs, 2007). The clinical correlates of this cholinergic crisis are hypersalivation, bronchorrhea, bronchoconstriction, convulsions, neuromuscular block, and finally respiratory arrest and death (Marrs, 2007; Grob, 1956).

Since decades, the standard treatment of OP nerve agent poisoning comprises the muscarinic antagonist atropine and an AChE reactivator, i.e., oximes like obidoxime or pralidoxime (Cannard, 2006; Johnson et al., 2000; Thiermann et al., 2013). However, numerous studies demonstrated the limited effect of standard nerve agent treatment, especially in poisoning by the nerve agents soman, tabun, and cyclosarin (Worek and Thiermann, 2013; Newmark, 2004).

In consequence, ongoing research is conducted to minimize or to prevent systemic nerve agent effects (Doctor et al., 1991; Lenz et al., 2007). Most research efforts are directed to enzyme-based scavengers that either covalently capture via a 1:1 molar ratio (stoichiometric) or hydrolyse in a catalytic manner nerve agents before they can attack synaptic AChE (Masson and Rochu, 2009; Mumford et al., 2013). At present, human butyrylcholinesterase (BChE), a stoichiometric scavenger, is the lead candidate and showed efficacy after pre- and post-exposure use (Brandeis et al., 1993 Allon et al., 1998; Mumford and Troyer, 2011). Major disadvantages of human BChE are the high production costs and the need to administer hundreds of milligrams to detoxify lethal nerve agent concentrations (Ashani and Pistinner, 2004; Allon et al., 1998).

An alternative approach is based on the identification and optimization of catalytic bioscavengers (Masson and Rochu, 2009). Candidate enzymes include mammalian paraoxonase (PON1), *Pseudomonas diminuta* organophosphorus hydrolase (OPH), *Alteromonas* prolidase organophosphorus acid anhydrase (OPAA) and *Loligo vulgaris* diisopropylfluorophosphatase (DFPase).

Albeit, the existing, wild-type enzymes preferentially hydrolyse the less toxic P(+) nerve agent enantiomers, and thus, are incapable of detoxifying nerve agents at an adequate rate (Masson and Rochu, 2009; diTargiani et al., 2010; Otto et al., 2013). Indeed, previous analysis indicated that effective detoxification at reasonable enzyme doses ($\leq$1 mg kg^{-1} body weight, assuming an enzyme M.W. of ~40 KDa) demands a catalytic efficiency (k_{cat}/K_M) of >10^7 M^{-1} min^{-1} (Ashani et al., 2011). This challenge directed research efforts to engineer enzyme mutants with P(−) stereopreference and sufficiently high k_{cat}/K_M (Amitai et al., 2006; Gupta et al., 2011).

Recently, recombinant serum paraoxonase (PON1) mutants (rePON1) with >1000-fold preferential hydrolysis of the toxic P(−) enantiomers of G-type nerve agents, i.e., soman and cyclosarin, were evolved. The efficacy of one of these mutants, rePON1 mutant IIG1, was demonstrated for prophylactic protection in cyclosarin poisoned guinea pigs (Goldsmith et al., 2012; Worek et al., 2014).

V-type nerve agents, i.e., VX and Russian VX (RVX), comprise a specific challenge for the development of catalytic scavengers (Masson and Rochu, 2009). These phosphonothiolates are barely hydrolysed by natural enzymes (Reeves et al., 2008; Bigley et al., 2013). Recently, Cherny et al. described engineered *Brevundimonas diminuta* phosphotriesterase (PTE) mutants with an increased detoxification rate of a variety of V-agents (Cherny et al., 2013). Specifically, some of these mutants hydrolysed the toxic P(−) enantiomers of VX, RVX, and Chinese VX (CVX) with k_{cat}/K_M values up to 5×10^6 M^{-1} min^{-1}, as measured *in vitro*.

These promising *in vitro* data prompted us to investigate the ability of post exposure treatment with the PTE mutant C23 to detoxify VX *in vivo* and to correlate the product of enzyme blood levels and k_{cat}/K_M with the manifestation of toxic signs. Guinea pigs were poisoned with a lethal VX dose and C23 was administered as a post-exposure therapy in the absence of any additional therapeutics. This first proof-of-concept study should allow an assessment of the ability of the tested PTE mutant and subsequently evolved variants to detoxify VX at a rate sufficient to prevent toxic signs of poisoning by low enzyme dose.

2. Materials and methods

2.1. Chemicals

The OP nerve agent VX, *O*-ethyl *S*-(2-diisopropylaminoethyl) methylphosphonothioate (>98% by GC–MS, ^{1}H NMR and ^{31}P NMR) was made available by the German Ministry of Defence. Triton X-100, tris[hydroxymethyl]-aminomethane (TRIS), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), ethopropazine and acetylthiocholine iodide (ATCh) were supplied by Sigma–Aldrich. All other chemicals were from Merck (Darmstadt, Germany).

VX stock solutions (1% v/v) were prepared in acetonitrile and were stored at ambient temperature. VX working solution (36 μg mL^{-1}) for *in vivo* testing was prepared for each experiment in saline and was kept on ice until use.

2.2. Enzyme expression and purification

The recombinant PTE variant C23 (MBP fusion of PTE_C23) was expressed as follows: The gene was cloned into a pMALc2x vector (NEB®) and transformed into *E.coli* BL21/DE3 cells. The culture grew in LB medium including ampicillin supplemented with 0.5 mM $ZnCl_2$ overnight at 30 °C. The inoculate was diluted 1:100 and grown at 30 °C to OD^{600} nm ≈ 0.6. IPTG was added (0.4 mM), and the culture was allowed to grow at 20 °C for 42 h. Cells were harvested by centrifugation and re-suspended in buffer A (PBS supplemented with 0.1 mM $ZnCl_2$). Cells were then lysed using sonication, clarified by centrifugation (20,000 rpm, 4 °C, 30 min) and passed through a column packed with amylose beads (NEB®) pre-equilibrated with buffer A. Following an extensive wash with buffer A, the MBP–PTE_C23 fusion protein was eluted with buffer A containing 10 mM maltose. The pooled fractions containing enzyme were further purified by anion exchange chromatography (IEX) on a HiPrep Q FF 16/10 (GE Healthcare®) equilibrated with 50 mM Tris pH 8. The protein was eluted with a linear gradient of NaCl (0–1 M over 5CV). The fractions containing pure MBP–PTE_C23 were pooled and dialyzed over night at 4 °C with an isotonic buffer (50 mM Tris pH 8, 100 mM NaCl and 10 μM $ZnCl_2$). The final yield was >300 mg protein (>95% pure) from a 7.5 L culture. Purity and protein concentrations were determined by SDS–PAGE and absorbance at 280 nm (extinction coefficient ε_{280} 95,925 M^{-1} cm^{-1}).

2.3. Animals

Male Dunkin–Hartley guinea pigs (350–400 g) were supplied by Charles River (Sulzfeld, Germany). The animals were kept under standard conditions (room temperature 20–22 °C, humidity 55%, 12 h light/dark cycle) and had free access to standard lab chow and water. Animals were allowed to accustom to the facility for at least one week before starting experiments. The experimental protocol was approved by the institutional ethics committee.

2.4. Experimental procedure

The guinea pigs were anesthetized by i.m. injection of a mixture of medetomidin (0.2 mg kg^{-1}), midazolam (1.0 mg kg^{-1}) and fentanyl (0.025 mg kg^{-1}). Anesthesia was continued throughout the observation period by additional injections of the anesthesia mix if required, i.e., one third of the initial dose in case of voluntary movements. Then, the animals were placed on a heatable operating table in supine position, a rectal thermistor was inserted and the body temperature was maintained at 37 °C. The right v.

jugularis and left a. carotis were prepared and catheters were inserted and fixed.

2.5. The animals were randomly divided into six groups:

- Saline control ($n = 6$; group C).
- C23 control (5 mg kg^{-1} i.v.; $n = 3$; group P).
- VX control (18 μg kg^{-1} s.c.; $n = 6$; group V).
- C23 (5 mg kg^{-1} i.v.) therapy 5 min post VX exposure ($n = 6$; group T1).
- C23 (2 mg kg^{-1} i.v.) therapy 5 min post VX exposure ($n = 4$; group T2).
- C23 (5 mg kg^{-1} i.v.) therapy 15 min post VX exposure ($n = 4$; group T3).

The control groups received saline (group C), C23 (5 mg kg^{-1} i.v.; group P) or VX (18 μg kg^{-1} s.c.; group V) at 0 min. In the therapy groups VX (18 μg kg^{-1} s.c.) was injected at $t = 0$ followed by C23 5 mg kg^{-1} i.v. (group T1) or 2 mg kg^{-1} i.v. (group T2) after 5 min and by C23 5 mg kg^{-1} i.v. (group T3) after 15 min. The injection volume was 0.5 mL kg^{-1} throughout. It should be noted that in view of the covalently attached maltose-binding protein (MBP) to the C23 template, the actual injected net enzyme is approximately half the listed doses above.

Multiple arterial blood samples (350 μL, the volume was replaced by saline) were taken in heparinized syringes between −1 and +180 min for the measurement of AChE activities, C23 and VX levels. Hereby, 50 μL whole blood were immediately diluted 20 fold in ice-cold distilled water, vortex mixed, shock frozen in liquid nitrogen, and stored at −80 °C until measurement of AChE activity. 200 μL whole blood were transferred into 600 μl formate buffer (VX analysis) and 100 μL blood were centrifuged (1 min, 13,000 rpm), the plasma was removed, shock frozen in liquid nitrogen and stored −80 °C until measurement of C23 concentration.

At 180 min or death of the animal the brain was removed, immediately dissected into frontal cortex, striatum, hippocampus and medulla, shock frozen in liquid nitrogen, and stored at −80 °C until measurement of AChE activity.

Clinical signs and symptoms were visually recorded and scored into local fasciculations at injection site, salivation/bronchorrhea, local or generalized convulsions, altered respiration (labored breathing), respiratory depression (frequency, rhythm, gasping), and death.

2.6. AChE assay

Brain tissue was mixed with a 10-fold volume of phosphate buffer (0.1 M, pH 7.4) containing 1% Triton X-100 and was homogenized in a glass–teflon Potter (Braun Melsungen, Darmstadt, Germany) on ice. The homogenates were centrifuged (Hettich Microfuge 22) at maximum speed for 1 min and the supernatant was used for the AChE assay.

Brain and erythrocyte AChE activities were measured with a modified Ellman assay (Worek et al., 1999) at 436 nm (Cary 50, Varian, Darmstadt) using polystyrol cuvettes, 0.45 mM ATCh as substrate, 0.02 mM ethopropazine as selective BChE inhibitor and 0.3 mM DTNB as a chromogen in 0.1 M phosphate buffer (pH 7.4). Erythrocyte AChE activity was referred to the hemoglobin concentration of the individual blood dilution, determined with the cyanmethemoglobin method (Worek et al., 1999), and was expressed as mU/μmol Hb while brain AChE activity was calculated as mU/mg wet weight.

2.7. C23 analysis

C23 levels in plasma were determined by hydrolysis of 3-cyano-7-hydroxy-4-methylcoumarin (CMP) as previously described (Gupta et al., 2011). Briefly, the initial velocity was monitored at 400 nm following 20 μL plasma dilution in 1 mL 50 mM TRIS–HCl pH 8.0 plus 50 mM NaCl, containing 100 μM CMP. A pre-determined calibration curve generated by adding increasing amounts of C23 to equal volume of assay sample containing 20 μL of naïve animal plasma, was used to calculate

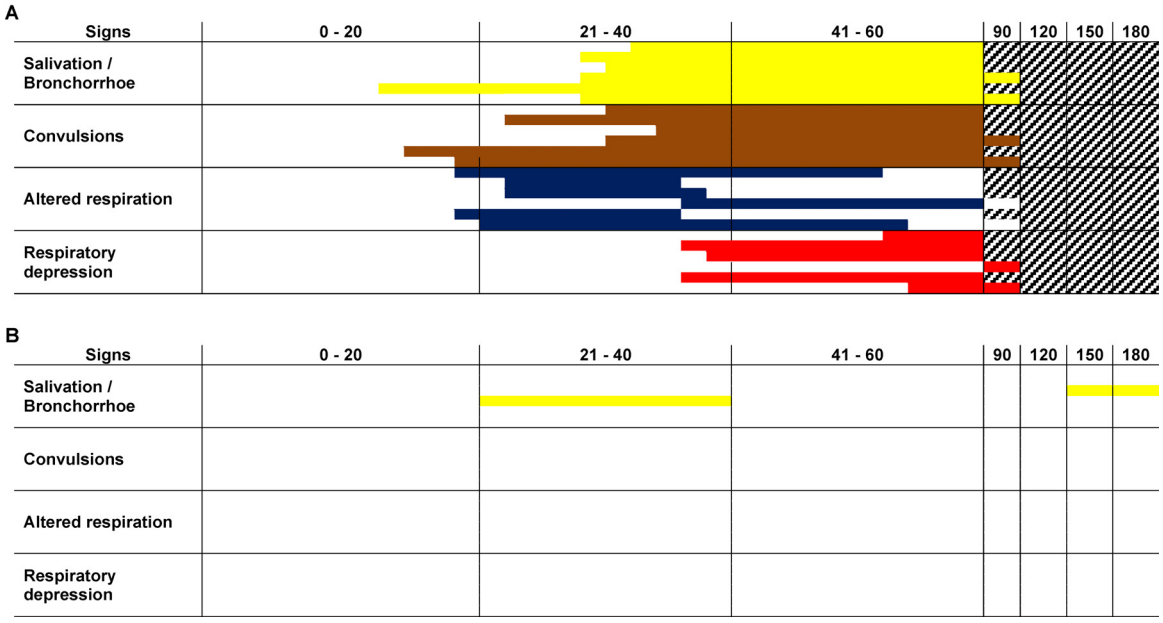

Fig. 1. Clinical signs and survival of VX-poisoned guinea pigs and effect of post-exposure treatment with C23. Each line represents a single animal and the time is given in minutes. The signs are subdivided into salivation/bronchorrhoe, convulsions (local or generalized), altered respiration (labored breathing) and respiratory depression (frequency, rhythm, gasping). Dead animals are marked by hatched lines. (A) 18 μg kg^{-1} VX s.c. (group V), (B) 18 μg kg^{-1} VX s.c. followed by 5 mg kg^{-1} C23 i.v. after 5 min (group T1).

C23 in plasma samples. Guinea pigs plasma is devoid of CMP hydrolytic activity.

2.8. Analysis of VX in whole blood samples

The quantification of racemic VX and VX enantiomers was performed by LC–MS-MS as described before with slight modifications (Reiter et al., 2008, 2011). 200 μL guinea pig whole blood were mixed with 600 μL 50 mM sodium formate buffer (pH 3.75) and incubated on ice for 1 min before adding 400 μL 100 mM sodium formate buffer (pH 3.75). All samples were immediately frozen in liquid nitrogen and then stored at −80 °C until further analysis.

Immediately prior to sample preparation the samples were thawed and spiked with internal standard (IS; *O*-isobutyl *S*-[2 (diethylamino) ethyl] methylphosphonothioate) at a final concentration of 100 pg mL^{-1}. For analysis of VX enantiomers 66.6 mL of 2-propanol were added to each 1 mL sample for protein precipitation and for analysis of racemic VX 5 mL of mixture 2-propanol, hexane and methanol 1:1:1 were added to each 1 mL sample. After centrifugation (4000 × *g*, 4 °C, 15 min) the supernatant was transferred to a Turbo Vap LV work station and the organic phases were evaporated at 40 °C with nitrogen (25 psi) up to 1 mL. Then, 10 mL of 2-propanol were added to each sample and the evaporation was carried out up to 1 mL again. Addition of 2-propanol and evaporation was repeated two times. Subsequently, 1 mL of tetradecane was added to each sample and the evaporation was continued up to "dry" tetradecane. Then, the reconstitution was carried out by adding 1 mL distilled water. After centrifugation (8200 × *g*, 40 min at 0 °C) a defined volume of the intermediate phase (0.6–0.7 mL) was diluted 1:1 with distilled water and transferred to an autosampler vial for LC–MS-MS analysis.

The analytical LC–MS/MS system for the quantification of racemic VX and VX enantiomers consisted of Prominence 20HPLC (Shimadzu, Duisburg, Germany) and a triple quadrupole 4000 Q Trap mass spectrometer as detector (AB SCIEX, Darmstadt, Germany). Following materials and LC/MS conditions were used:

- Columns: CHIRAL AGP (150 mm × 2 mm i.d., 5 μm particle size, Regis Technologies, USA) for quantification of VX enantiomers (E) and HYPERCARB for quantification of racemic VX (R) (150 mm × 2.1 mm i.d., 5 μm particle size, Thermo Fisher Scientific, Germany).
- Flow rates: 150 μL min^{-1} (E) and 140 μl min^{-1} (R).
- Injection volume 500 μL and the column temperature 30 °C (E and R).
- Mobile phase: 50 mM ammonium formate buffer pH 9.0–methanol (1:1, eluent A) and 25 mM ammonium formate buffer pH 9.0 (eluent B) (E); methanol (eluent A) and 25 mM ammonium formate buffer pH 5.0 (eluent B) (R).
- Linear gradients: 7 min rinse with distilled water (E and R); A from 0% to 37.5% within 49 min, maintained for 15.5 min, re-equilibration to 0% A within 3.5 min and maintained for 5 min (E); rinse 7–11 min with 25% A, from 25% to 36% A within 6.5 min, from 36% to 60% A within 2.5 min, maintained for 7.5 min, re-equilibration to 25% A within 3.5 min and maintained for 7 min (R).

MS (positive electrospray ionization) conditions were as follows:

- curtain gas (25 psi), collision gas (setting medium), ion source gas 1 (30 psi) and ion source gas 2 (50 psi) (E and R), the interface temperature 400 °C (E) and 300 °C (R), the ion spray voltage 5.5 kV, the declustering, entrance, collision cell exit potentials and collision energy 38, 10, 8 and 30 V, respectively (E and R).
- multiple reaction monitoring (MRM) mode, precursor $[M+H]^+$ → product ion mass transitions: m/z 268.4 →128.2 for VX and m/z 268.4 →100.2 for IS, dwell-time 2 s per transition (E and R).

2.9. Data analysis

Data are presented as mean ± standard deviation (SD). Statistical comparisons were performed using Graph-Pad Prism Version 4.03 (GraphPad Software, San Diego, CA, USA). Differences of brain AChE activities between groups were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. Concentration of racemic VX and erythrocyte AChE activity differences between and within groups were analyzed by two-way ANOVA and Bonferroni post-tests. A $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. VX poisoning

The subcutaneous injection of 18 μg kg^{-1} VX (~$2LD_{50}$) (Shih et al., 2011; Wetherell et al., 2006) resulted in a rapid onset of local fasciculations which became already visible during the slow (1 min) injection of VX. Signs of cholinergic overstimulation, i.e., salivation,

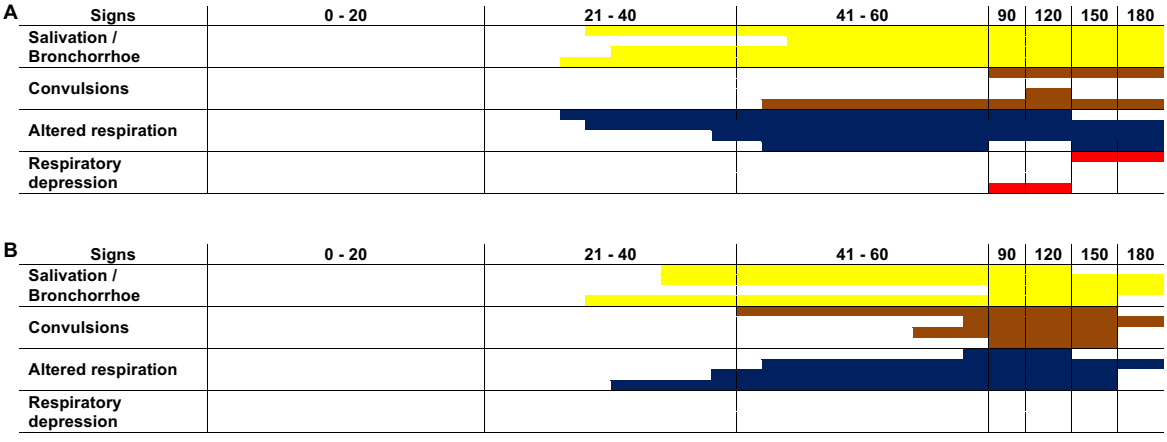

Fig. 2. Clinical signs and survival of VX-poisoned guinea pigs with post-exposure C23 treatment. Each line represents a single animal and the time is given in minutes. The signs are subdivided into salivation/bronchorrhoe, convulsions (local or generalized), altered respiration (labored breathing) and respiratory depression (frequency, rhythm, gasping). (A) 18 μg kg^{-1} VX s.c. followed by 2 mg kg^{-1} C23 i.v. after 5 min (group T2), (B) 18 μg/kg VX s.c. followed by 5 mg kg^{-1} C23 i.v. after 15 min (group T3).

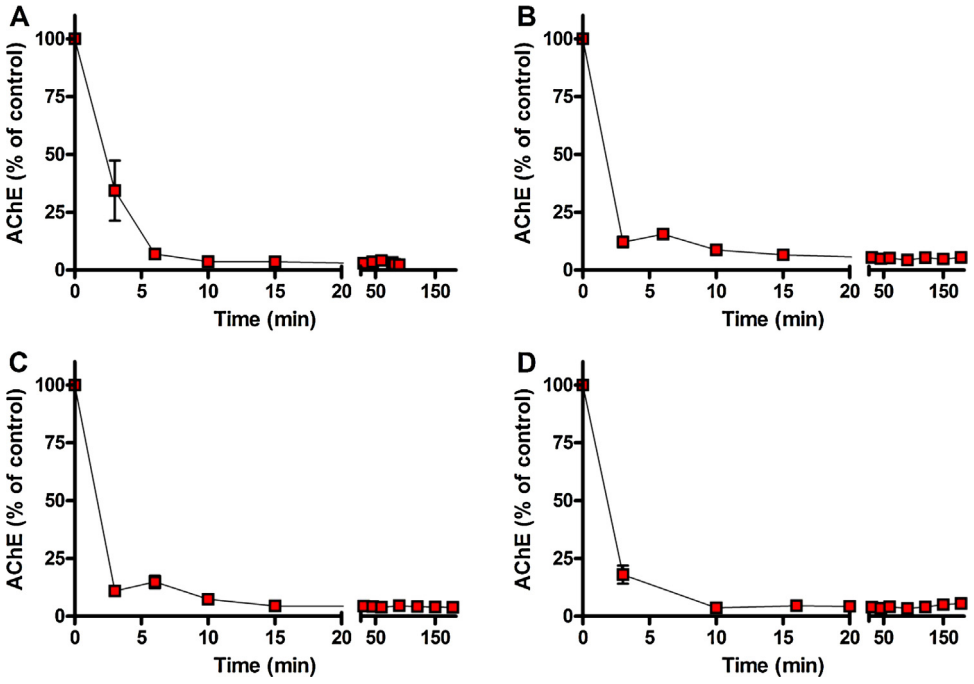

Fig. 3. Time-dependent changes of erythrocyte AChE activity. (A) 18 μg kg^{-1} VX s.c. (group V), (B) 18 μg kg^{-1} VX s.c. followed by 5 mg kg^{-1} C23 i.v. after 5 min (group T1), (C) 18 μg kg^{-1} VX s.c. followed by 2 mg kg^{-1} C23 i.v. after 5 min (group T2) and (D) 18 μg kg^{-1} VX s.c. followed by 5 mg kg^{-1} C23 i.v. after 15 min (group T3). Data are given as % of pre-exposure control AChE activity as means ± SD.

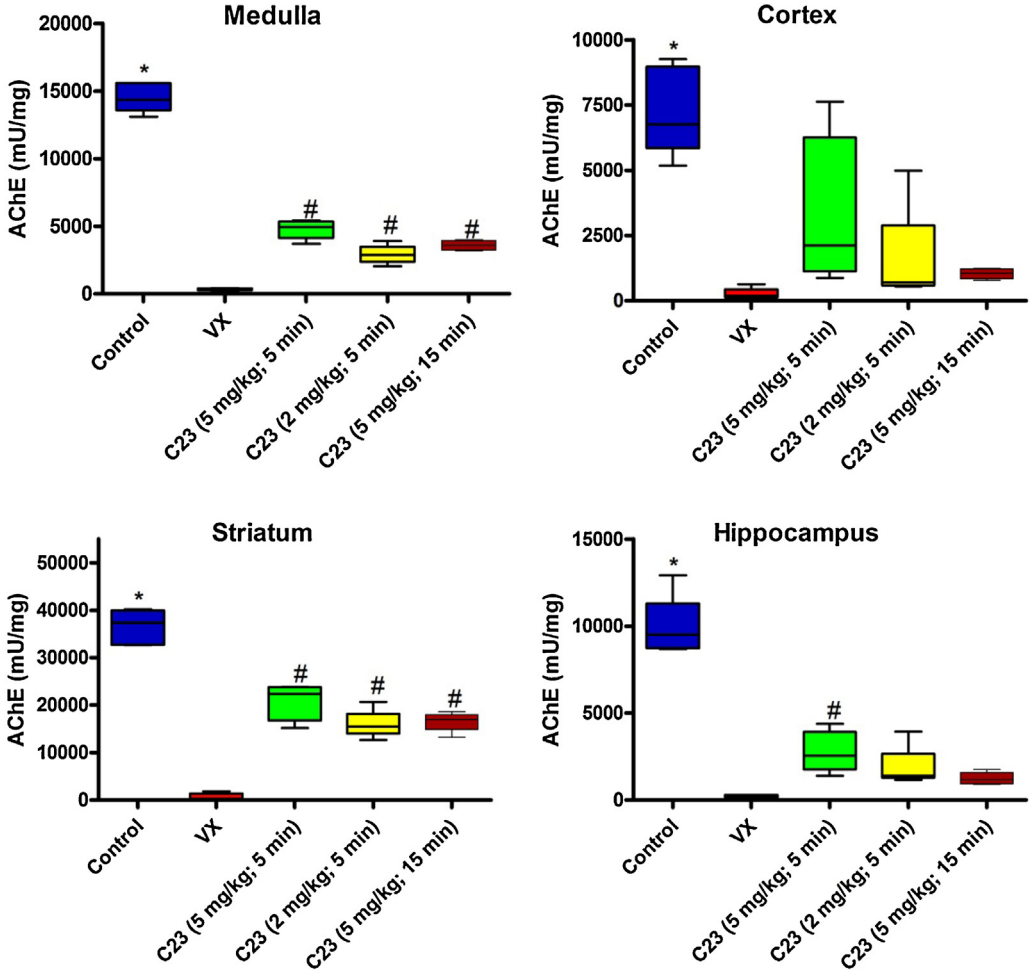

Fig. 4. Brain AChE activity of control, VX and C23 treated guinea pigs. Data of medulla, cortex, striatum and hippocampus AChE activity (mU/mg) are given as means ± SD. $^{*}p < 0.05$ versus VX and therapy groups; $^{\#}p < 0.05$ versus VX group.

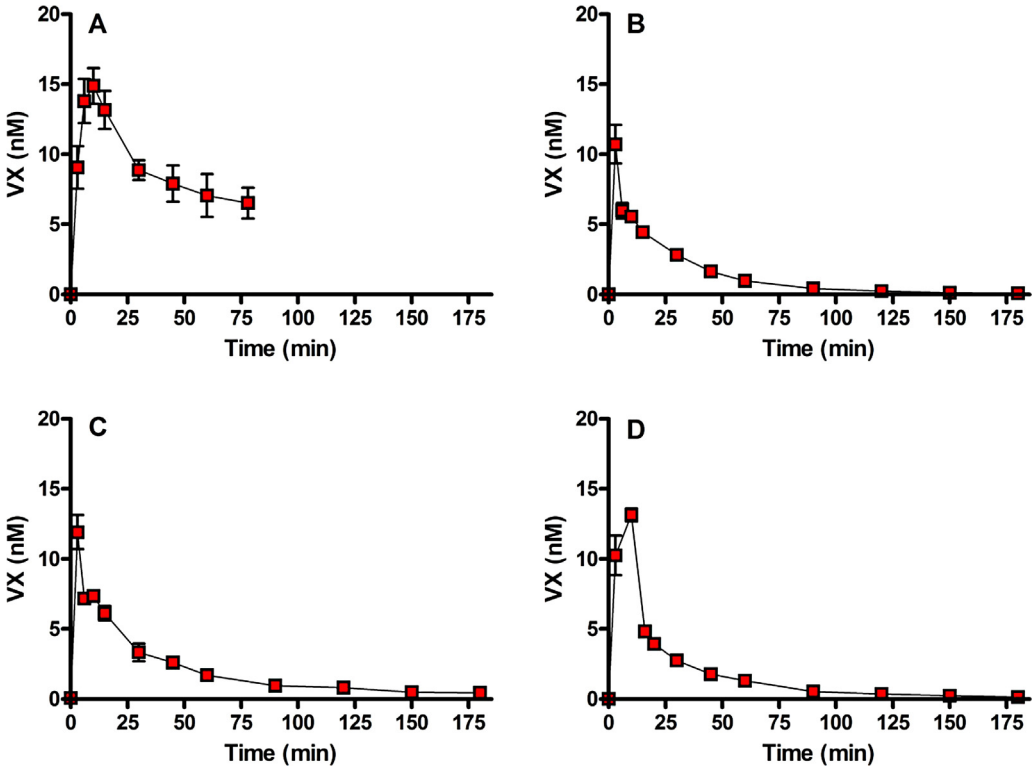

Fig. 5. Whole blood concentration of racemic VX. (A) 18 μg kg^{-1} VX s.c. (group V), (B) 18 μg kg^{-1} VX s.c. followed by 5 mg kg^{-1} C23 i.v. after 5 min (group T1), (C) 18 μg kg^{-1} VX s.c. followed by 2 mg kg^{-1} C23 i.v. after 5 min (group T2) and (D) 18 μg kg^{-1} VX s.c. followed by 5 mg kg^{-1} C23 i.v. after 15 min (group T3). Data are given in nmol L^{-1} whole blood as means ± SD. VX concentrations of therapy groups T1 and T2 were significantly lower ($p < 0.05$) compared to VX group from 6 min on and to group T3 from 16 min on.

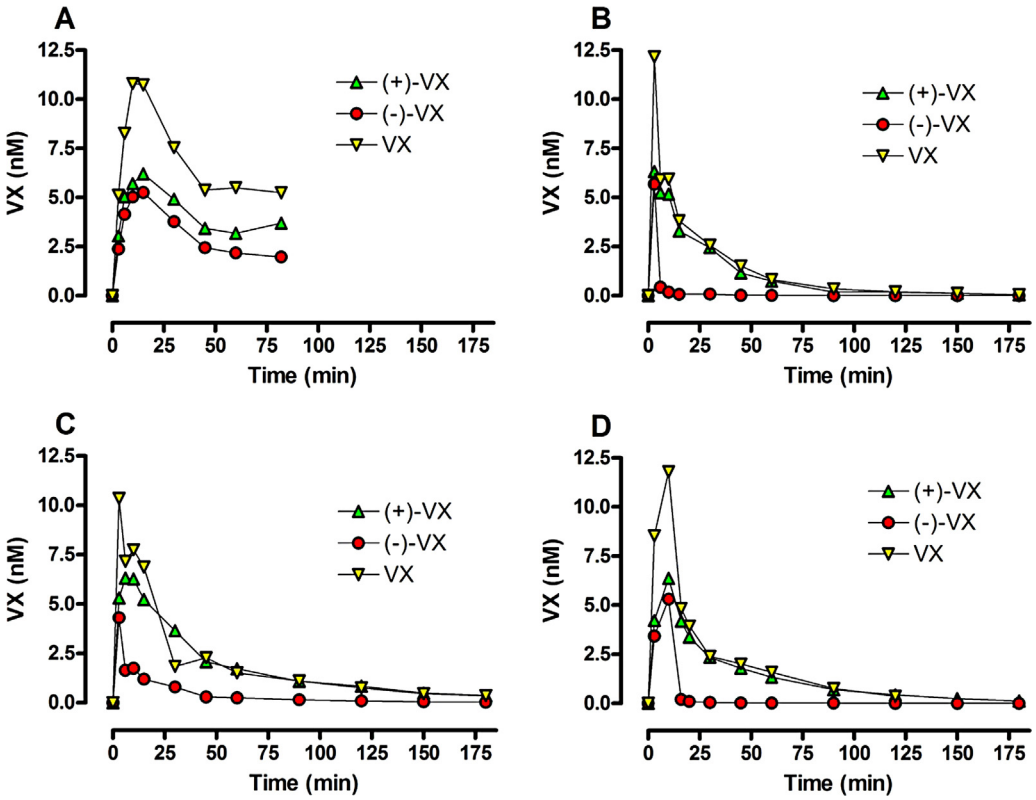

Fig. 6. Concentration of VX enantiomers in whole blood samples of individual animals. (A) 18 μg kg^{-1} VX s.c. (group V), (B) 18 μg kg^{-1} VX s.c. followed by 5 mg kg^{-1} C23 i.v. after 5 min (group T1), (C) 18 μg kg^{-1} VX s.c. followed by 2 mg kg^{-1} C23 i.v. after 5 min (group T2) and (D) 18 μg kg^{-1} VX s.c. followed by 5 mg kg^{-1} C23 i.v. after 15 min (group T3). Data are given in nmol L^{-1}.

bronchorrhea, clonic convulsions of the lower limbs followed by generalized convulsions and labored breathing developed within 20–30 min (Fig 1A). The signs aggravated with time and were followed by respiratory depression, i.e., reduced frequency, deep breathing and gasping, and finally respiratory arrest in all animals. The animals died between 63 and 107 min after VX injection (85 ± 13 min).

3.2. C23 treatment

The engineered PTE variant C23 was tagged with maltose-binding-protein (MBP) at its N-terminus, and purified and injected as such. Given the relative molecular weights (MBP + linker, 42.5 KDa; PTE-C23, 36.7 KDa), the effective enzyme dose comprised a fraction of ~0.46 of the injected protein dose. Treatment of VX poisoned guinea pigs with 5 mg kg^{-1} i.v. C23 5 min after VX administration (group T1) resulted in survival of all treated animals and prevented almost all signs of poisoning (Fig. 1B). Local fasciculations at the injection site were observed in all animals until the end of the observation period and only two guinea pigs suffered from transient salivation.

Administration of a reduced C23 dose (2 mg kg^{-1} i.v.; group T2) 5 min after VX delayed the onset of signs of poisoning (Fig. 2A). All animals experienced salivation/bronchorrhea and labored breathing, 3 animals developed moderate convulsions and 2 animals suffered from transient respiratory depression. All animals survived until end of the observation period.

Finally, the delayed injection of C23 (5 mg kg^{-1} i.v.; group T3), i.e., 15 min after VX, prevented death and reduced the toxic effects of VX (Fig. 2B). All animals developed moderate cholinergic signs, salivation/bronchorrhea, labored breathing and convulsions.

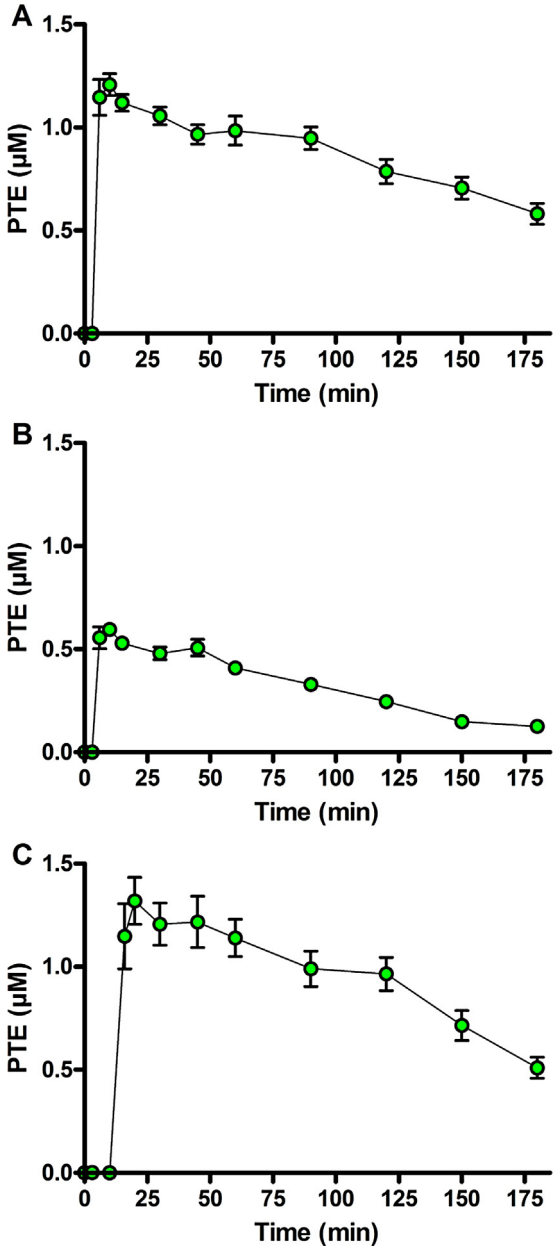

Fig. 7. C23 plasma concentration profile. (A) 18 μg kg^{-1} VX s.c. followed by 5 mg kg^{-1} C23 i.v. after 5 min (group T1), (B) 18 μg kg^{-1} VX s.c. followed by 2 mg kg^{-1} C23 i.v. after 5 min (group T2) and (C) 18 μg kg^{-1} VX s.c. followed by 5 mg kg^{-1} C23 i.v. after 15 min (group T3). Data are given in μmol L^{-1} plasma as means ± SD.

3.3. Erythrocyte AChE activity

The pre-exposure erythrocyte AChE activities were 168 ± 21 mU μmol^{-1} Hb (group C), 169 ± 32 mU μmol^{-1} Hb (group V), 167 ± 18 mU μmol^{-1} Hb (group T1), 160 ± 26 (group T2) and 165 ± 23 mU μmol^{-1} Hb (group T3).

VX poisoning resulted in a rapid inhibition of erythrocyte AChE activity reaching almost zero levels after 10 min (Fig. 3A). Administration of C23 5 min after VX was able to delay the inhibition of erythrocyte AChE slightly but could not prevent complete inhibition (Fig. 3B + C). Delayed treatment with C23 (group T3) had no protective effect on erythrocyte AChE activity (Fig. 3D).

3.4. Brain AChE activity

VX poisoning resulted in a complete inhibition of AChE activity in medulla oblongata, frontal cortex, striatum, and hippocampus (Fig. 4). Treatment with 5 mg kg^{-1} C23 (5 min; group T1) protected brain AChE activity partially which was significantly higher in medulla, striatum, and hippocampus compared to the VX group (Fig. 4). Post-exposure treatment with the lower C23 dose (2 mg kg^{-1}; 5 min; group T2) as well as delayed treatment (group T3) provided some protection (Fig. 4). However, brain AChE activity of therapy groups T1, T2 and T3 was significantly lower compared to saline control group.

3.5. VX concentration

The analysis of racemic VX concentrations in whole blood samples showed a rapid increase of VX reaching a maximum whole blood concentration of ~15 nmol L^{-1} in the VX control group (Fig. 5). There was no significant difference in the initial (3 min) increase of VX concentration but a significant, C23 induced, decrease of VX in the therapy groups. VX concentrations of less than 1 nM were determined in therapy group T1 at 60 min and in therapy groups T2 and T3 at 90 min.

In addition, the concentration of individual VX enantiomers, (−)-VX and (+)-VX, was analyzed. As an example, Fig. 6 shows results from individual animals. In VX treated control animals a concurrent course of both VX enantiomers was recorded resulting in a (−)-VX concentration of ~2 nM at time of death (Fig. 6A). Treatment of VX poisoned guinea pigs with 5 mg kg^{-1} C23 5 min after VX caused a rapid decrease of (−)-VX resulting in a concentration of ~0.7 nM 1 min, and of <0.1 nM 30 min after C23 injection (Fig. 6B). Treatment with a lower C23 dose, 2 mg kg^{-1}, resulted in a slower decrease of (−)-VX (Fig. 6C). (−)-VX concentrations of less than 1 nM and 0.1 nM were reached after 15 and 120 min, respectively. Finally, the injection of 5 mg kg^{-1} C23

15 min after VX resulted in a rapid decrease of (−)-VX to less than 1 nM within 1 min (Fig 6D).

3.6. C23 plasma concentration

The i.v. injection of 2 and 5 mg kg^{-1} C23, respectively, resulted in a dose-dependent maximum plasma concentration of ~0.6 and ~1.3 μmol L^{-1}, as determined by the levels of serum enzyme activities (Fig. 7), and was followed by a slow decrease until the end of the observation period.

Intravenous injection of 5 mg kg^{-1} C23 (group P) had no effect on erythrocyte and brain AChE activity and did not result in any clinical signs (data not shown).

4. Discussion

To the best of our knowledge, this is the first study that demonstrates a successful protection of experimental animals against VX intoxication by use of a catalytic bioscavenger that was administered in a post-exposure modality.

Thus, this proof-of-concept study showed that a catalytic bioscavenger with a k_{cat}/K_M of 5×10^6 M^{-1} min^{-1} is able to prevent systemic toxicity of the highly toxic nerve agent VX *in vivo* at low protein dose. VX is characterized by a high inhibitory potency towards AChE from different species, i.e., having a second order inhibition rate constant k_i of 1.15×10^8 M^{-1} min^{-1} with human AChE and of 8.2×10^7 M^{-1} min^{-1} with guinea pig AChE (Worek and Thiermann, 2013). In addition, VX shows a high biological persistence *in vivo*, especially after percutaneous exposure (van der Schans et al., 2003; Reiter et al., 2011; Wetherell et al., 2008). Furthermore, the concentration profile of P(−)- and P(+)-VX enantiomers in swine is comparable indicating a negligible degradation by endogenous enzymes (Reiter et al., 2008). In contrast to rapidly eliminated G-type nerve agents (Benschop and de Jong, 2001; Tenberken et al., 2010; Reiter et al., 2007) the toxicokinetic profile of VX requires the long-term administration of antidotes, e.g., injection of multiple oxime doses, to preserve a fraction of active AChE and to prevent lethality (Joosen et al., 2010).

Wild-type PTE has a negligible catalytic efficacy against V-type nerve agents (Masson and Rochu, 2009; Kolakowski et al., 1997). Indeed, the k_{cat}/K_M value for hydrolysis of the toxic VX isomer (the P(−) or S_P isomer) by wild-type PTE is ~10^4 M^{-1} min^{-1} (Cherny et al., 2013). Extensive research efforts are undertaken to engineer sufficiently active PTE mutants (Bigley et al., 2013; Tsai et al., 2010; Jeong et al., 2014). Recently, Cherny et al. described PTE mutants with ~500-fold improved catalytic efficiency (k_{cat}/K_M) for hydrolysis of V-type nerve agents, and specifically, for hydrolysis of the toxic P(−) enantiomers (Cherny et al., 2013). Here, the leading candidate, PTE mutant C23, was tested for the first time against VX *in vivo*, and the data of the present study demonstrate its ability to prevent systemic toxicity in guinea pigs.

The subcutaneous injection of a lethal VX dose resulted in a rapid increase of VX in blood (Fig. 5) reaching concentrations of up to 15 nM at the first analysis time point, i.e., at 3 min post VX. Despite the rapid resorption from the injection site and distribution of VX into the systemic circulation, the development of systemic signs of poisoning was delayed to 20 to 30 min (Fig. 1A) while subcutaneous injection of cyclosarin, sarin, and soman resulted in a substantially faster onset of systemic signs (Bueters et al., 2002; Wetherell et al., 2002; Worek et al., 2014). This observation indicates a delayed distribution of VX into tissue which may be due to the fact that VX (pK_a ~8.5) circulates in part as a protonated amine (Shih et al., 2005; Epstein et al., 1974). In fact, previous studies in guinea pigs with percutaneous VX exposure revealed a slow increase of VX concentration in blood and with delay in tissue, a slow decrease of AChE and BChE activities accompanied by a delayed onset of signs of poisoning (Mumford et al., 2011, 2013; van der Schans et al., 2003). Mumford et al. nicely showed that post-exposure therapy of guinea pigs poisoned by percutaneous VX application with 72 mg kg^{-1} of the stoichiometric bioscavenger human BChE is effective if given prior to onset of signs of poisoning but loses effectiveness dramatically if given at appearance of first signs of intoxication (Mumford et al., 2011, 2013; Mumford and Troyer 2011).

In the present study, the subcutaneous VX injection resulted in a more strict toxicokinetics and condensed timeline, i.e., first signs of poisoning in the VX control group were within 15–20 min (Fig. 1). Hence, the window of opportunity for administration of the catalytic bioscavenger was substantially shorter. In fact, injection of C23 at 5 min after VX resulted in rapid elimination of P(−)-VX in blood (Fig. 6) but could not prevent partial inhibition of brain AChE activity (Fig. 4) which indicates that VX penetrated the blood brain barrier within the initial 5–10 min. This view is supported by the fact that delayed, i.e., 15 min post VX, C23 injection induced again a rapid decrease of P(−)-VX concentration in blood but did not prevent a significant inhibition of brain AChE activity and the development of systemic signs of poisoning. Hence, the results of the present study affirm previous findings with stoichiometric bioscavengers and give a clear indication that post-exposure administration of bioscavengers must be carried out prior to the development of systemic signs of poisoning in order to confer reasonable protection against VX intoxication.

The estimated minimal k_{cat}/K_M for a catalytic bioscavenger assuming a dose of 1 mg enzyme per kg body weight is 10^7 M^{-1} min^{-1} (for an enzyme with an average molecular weight of 40 KDa) (Gupta et al., 2011; Josse et al., 2001). The *in vitro* measured k_{cat}/K_M for PTE mutant C23 is 5×10^6 M^{-1} min^{-1} with the toxic P(−)-VX enantiomer (Cherny et al., 2013). With a C23 peak plasma concentration of 1.15 μM (5 mg kg^{-1} of the MBP fusion of C23; Fig. 8A) the enzyme should hydrolyze more than 96% of P(−)-VX within 36 s [$t_{1/2} = 0.69/(5 \times 10^6 \times 1.15 \times 10^{-6}) = 7.25$ s] and at a lower C23 dose (2 mg kg^{-1}; Fig. 8B) within 75 s. The example given in Fig. 6B (5 mg kg^{-1} C23 injected 5 min after VX) is in good agreement with the theoretical calculation, i.e., decrease of P(−)-VX by ~93% within 1 min. In an animal treated with 2 mg kg^{-1} C23 the decrease of P(−)-VX was delayed (Fig. 6C) which may be due to an ongoing absorption of VX from the subcutaneous depot. The principles of prediction of *in vivo* efficacy of bioscavengers, both stoichiometric and catalytic, from detoxification *in vitro* (Ashani and Pistinner, 2004; Worek et al., 2014) are supported by the current study. Hence, it may be assumed that early post-

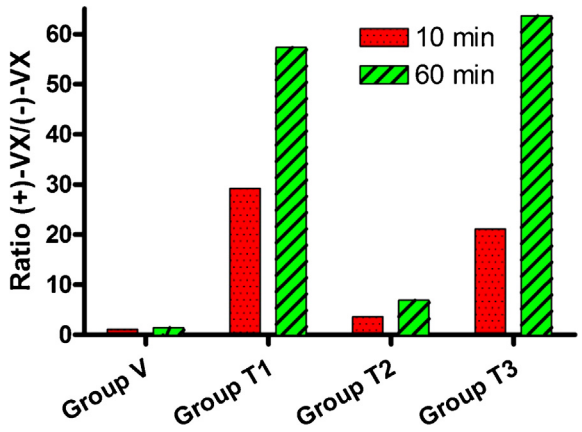

Fig. 8. Ratio of (+)VX and (−)VX 10 and 60 min after poisoning in whole blood samples of individual animals. Data shown are based on results presented in Fig. 6.

exposure administration of C23 should detoxify P(−)-VX at a sufficient rate to prevent toxicity of even higher VX doses.

According to our model, and the *in vitro* measured k_{cat}/K_M value, PTE mutant C23 should be further improved by ~8-fold to qualify as promising candidate in protecting against ≥2LD_{50} VX doses, and at a maximal dose of 1 mg enzyme per kg body weight (Cherny et al., 2013). *In vivo*, an almost exclusive degradation of the more toxic P(−)-VX enantiomer was observed (Fig. 6) resulting in ratios of P(+)/P(−) concentrations of ~25 at 10 min after C23 injection and of ~60 after 60 min at a C23 dose of 5 mg kg^{-1} (Fig. 8). In an animal treated with 2 mg kg^{-1} C23, the ratio was markedly low, i.e., 4 and 7 after 10 and 60 min, respectively. Probably, an ongoing resorption of VX together with the lower C23 dose caused this difference.

Post-exposure therapy with C23 at best slowed down the complete inhibition of erythrocyte AChE activity (Fig. 3). Due to the rapid resorption of VX from the subcutaneous injection site racemic VX concentrations of 10–12 nM were determined at 3 min (Fig. 5). At such a VX concentration the half-time of AChE inhibition would be less than 1 min according to $t_{1/2} = 0.69/(k_i \times [VX])$ with k_i 8.2×10^7 M^{-1} min^{-1} and [VX] 10 nM. These and previous results (Mumford et al., 2013) portend to the ability of blood cholinesterases to act as endogenous scavengers and demonstrate the suitability of erythrocyte AChE as a sensitive diagnostic parameter in poisoning by VX and other nerve agents. In addition, these data indicate that under the conditions of the applied model, i.e., absent equilibrium between blood and tissue VX concentration, erythrocyte AChE activity does not necessarily correlate with tissue AChE activities.

The PTE mutant C23 is a bacterial enzyme and a previous study showed rapid clearance of a wild-type Pseudomonas sp. PTE (Ashani et al., 1991). However, structural modifications in C23 apparently enhanced its longevity in the circulation and its plasma levels decreased rather slowly from initial 1.15 μM to 0.58 μM at 180 min (Fig. 7A) indicating a slow clearance by endogenous mechanisms. Notably, C23 peaked in guinea pigs plasma at more than 90% of the estimated value, assuming approximately 40 mL plasma/kg. A comparable concentration profile was observed with the free C23 enzyme in pilot experiments (data not shown). Nevertheless, a long life-time of bioscavengers is a prerequisite for successful use, especially in case of percutaneous poisoning by persistent nerve agents, and will require additional investigations to diminish the *in vivo* clearance of candidate bioscavengers.

In conclusion, this proof-of-concept study demonstrated for the first time the ability of a catalytic bioscavenger to prevent systemic VX toxicity *in vivo* if given as a single post-exposure treatment. This study was undertaken in anesthetized guinea pigs and the efficacy of C23 should be investigated in a conscious animal model as well, although a decisive effect of anesthesia is not expected. A remarkable good agreement of *in vitro* and *in vivo* VX degradation kinetics was demonstrated and therefore it is envisaged that increasing k_{cat}/K_M via molecular design should provide variants that will provide post exposure protection at well below 1 mg kg^{-1} protein dose. Notably, when a stoichiometric scavenger was employed to counteract VX intoxication via the percutaneous route, 72 mg kg^{-1} human BChE were administered to demonstrate post exposure protection (Mumford et al., 2011), which is approximately 30-fold greater than the protein dose utilized in this study. In addition, the necessity to administer the bioscavenger prior to the development of systemic signs of poisoning was demonstrated. The PTE mutant C23 may be considered as a promising template for the development of highly effective catalytic bioscavengers suitable for the post-exposure prophylaxis of VX and other biologically stable nerve agents.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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