

## Single treatment of VX poisoned guinea pigs with the phosphotriesterase mutant C23AL: Intraosseous versus intravenous injection



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### HIGHLIGHTS

- I.O. application of C23AL resulted in comparable plasma levels to I.V. application.
- I.M. application of C23AL did not result in detectable plasma levels within 3 h.
- C23AL showed lower breakdown of VX *in vivo* than calculated from *in vitro* data.
- Results underline necessity of *in vivo* experiments in antidote research.

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### ABSTRACT

The recent attacks with the nerve agent sarin in Syria reveal the necessity of effective countermeasures against highly toxic organophosphorus compounds. Multiple studies provide evidence that a rapid onset of antidotal therapy might be life-saving but current standard antidotal protocols comprising reactivators and competitive muscarinic antagonists show a limited efficacy for several nerve agents. We here set out to test the newly developed phosphotriesterase (PTE) mutant C23AL by intravenous (i.v.), intramuscular (i.m.; model for autoinjector) and intraosseous (i.o.; model for intraosseous insertion device) application in an *in vivo* guinea pig model after VX challenge (~2LD<sub>50</sub>).

C23AL showed a C<sub>max</sub> of 0.63 μmol L<sup>-1</sup> after i.o. and i.v. administration of 2 mg kg<sup>-1</sup> providing a stable plasma profile up to 180 min experimental duration with 0.41 and 0.37 μmol L<sup>-1</sup> respectively. The i.m. application of C23AL did not result in detectable plasma levels. All animals challenged with VX and subsequent i.o. or i.v. C23AL therapy survived although an in part substantial inhibition of erythrocyte, brain and diaphragm AChE was detected. Theoretical calculation of the time required to hydrolyze *in vivo* 96.75% of the toxic VX enantiomer is consistent with previous studies wherein similar activity of plasma containing catalytic scavengers of OPs resulted in non-lethal protection although accompanied with a variable severity of cholinergic symptoms. The relatively low C23AL plasma level observed immediately after its i.v. or i.o. load, point at a possible volume of distribution greater than the guinea pig plasma content, and thus underlines the necessity of *in vivo* experiments in antidote research.

In conclusion the i.o. application of PTE is efficient and resulted in comparable plasma levels to the i.v. application at a given time. Thus, i.o. vascular access systems could improve the post-exposure PTE therapy of nerve agent poisoning.

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## 1. Introduction

The recent use of the nerve agent sarin in Syria with several thousands dead or wounded civilians reveals the necessity of highly effective medical countermeasures against deadly organophosphorus compounds (OP) (Dolgin, 2013; Eisenkraft et al., 2014). OPs bind covalently to the pivotal enzyme acetylcholinesterase (AChE) rendering the enzyme in its inactive form (Aldridge and Reiner, 1972). This leads to an accumulation of the neurotransmitter acetylcholine with subsequent overstimulation of the cholinergic system at muscarinic and nicotinic synapses. The cholinergic crisis may ultimately result in death due to muscarinic receptor-mediated strong secretion in the airways, nicotinic receptor-mediated paralysis of the diaphragm and intercostal muscles and central respiratory disturbances (Marrs et al., 2007). Since 60 years, the standard antidotal treatment with the competitive muscarinic receptor antagonist atropine and an AChE reactivator, i.e. obidoxime or pralidoxime, is virtually unchanged and numerous studies provide evidence for limited efficacy against the nerve agents soman, tabun and cyclosarin (Worek and Thiermann, 2013). The current standard protocol is given after the first occurrence of signs and can thus only reactivate inhibited AChE after the OPs have already been distributed into target tissues and AChE inhibition is in progress.

In consequence, new approaches are directed to avoid distribution into target tissues and to prevent systemic toxicity by the administration of (enzyme-based) scavengers. As stoichiometric scavenger human butyrylcholinesterase (BChE) is under research and proofed feasibility for both prophylactic or therapeutic application (Allon et al., 1998; Mumford et al., 2013; Mumford and Troyer, 2011). Due to the stoichiometric binding and to its large molecular weight large BChE doses are necessary to detoxify nerve agents in the body resulting in high costs for the production of purified or recombinant BChE (Elsinghorst et al., 2013). In addition, remaining obstacles for recombinant protein production are potential immunogenicity and their inherent insufficient plasma stability.

To increase the turnover in nerve agent degradation catalytic bioscavengers have drawn interest of several research groups (Lenz et al., 2007; Masson and Rochu, 2009; Worek et al., 2016). Quite recently, research groups succeeded in generating enzyme mutants with a catalytic efficacy towards the toxic P(-) enantiomers of G-type nerve agents with  $k_{\text{cat}}/K_M$  values  $> 10^7 \text{ M}^{-1} \text{ min}^{-1}$  – a bench mark for effective detoxification at reasonable enzyme doses ( $< 1 \text{ mg kg}^{-1}$  body weight; assuming a molecular weight of the enzyme of 40 kDa) and a prospective use in humans (Gupta et al., 2011). The efficacy of a recombinant paraoxonase (PON1) mutant for prophylactic protection in cyclosarin poisoning was proofed in a guinea pig model (Goldsmith et al., 2012; Worek et al., 2014a). Moreover, Cherny et al. (2013) were successful in the design of *Brevundimonas diminuta* phosphotriesterase (PTE) mutants with  $k_{\text{cat}}/K_M$  values of up to  $5 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$  for the toxic P(-) enantiomer of V-agents *in vitro* which is an important milestone in development of potential medical countermeasures against V-type agent poisoning. This was proven *in vivo* in a single treatment study with the PTE mutant C23 resulting in reduced systemic toxicity and all animals surviving after s.c. challenge with  $2\text{LD}_{50}$  of VX (Worek et al., 2014b). From this promising starting point a further mutant with a higher activity *in vitro* ( $k_{\text{cat}}/K_M$  of  $1.2 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$  vs  $0.6 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ ) but a substantially lower molecular weight due to a missing stabilizing protein (36.9 vs 79.2 kDa) was generated. This PTE, C23AL, should allow the administration of substantial lower doses ( $\leq 2 \text{ mg kg}^{-1}$  body weight) and was subsequently tested as a tool to investigate different routes of PTE administration.

In severely poisoned patients with symptoms of shock or even cardiac arrest a peripheral intravenous (i.v.) access may not be readily available and delay the application of life-saving medical countermeasures (Anson, 2014). Furthermore, a time-consuming decontamination of poisoned patients is required to allow safe administration of i.v. drugs for both the patient and medical personnel. For this reason, intramuscular (i.m.) administration was performed with the PTE mutant C23AL as model for a rapid and easy application by e.g. an autoinjector as emergency therapeutic device in nerve agent poisoning (Thiermann et al., 2013). To rapidly gain vascular access the American Heart Association and European Resuscitation Council advocate intraosseous (i.o.) access as non-collapsible lumen of circulation with an equivalency to the i.v. application with commonly used emergency drugs (Anson, 2014; Neumar et al., 2010; Soar et al., 2015). In addition, even proteins with a large molecular mass  $> 60 \text{ kDa}$  have been successfully applied by the i.o. route in children and adults (Kelsall, 1993; Ruiz-Hornillos et al., 2011). As experiments with conventional chemical warfare antidotes (i.e. oxime and atropine) in Göttingen minipigs showed equivalent bioavailability compared to the intravenous route (Murray et al., 2012; Hill et al., 2015), we here set out to test the i.m. and i.o. application of C23AL as convenient techniques in disaster and emergency medicine and compared it to the standard i.v. line.

## 2. Materials and methods

### 2.1. Chemicals

The OP nerve agent VX, O-ethyl S-(2-diisopropylaminoethyl) methylphosphonothioate ( $> 98\%$  by GC-MS,  $^1\text{H}$  NMR and  $^{31}\text{P}$  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 ( $36 \mu\text{g}/\text{ml}$ ) 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 variants (MBP fusion) were purified as follows: The gene was cloned into a pMALc2x expression vector (New England BioLab) and transformed into *E. coli* GG48 cells. The culture grew in 2YT medium including ampicillin overnight at  $37^\circ\text{C}$ . The inoculate was dilute 1:100 into LB medium with ampicillin ( $100 \mu\text{g}/\text{ml}$ ) and  $0.2 \text{ mmol L}^{-1} \text{ ZnCl}_2$  and grown at  $37^\circ\text{C}$  to  $\text{OD}_{600\text{nm}} \approx 0.6$ . IPTG was added ( $0.4 \text{ mmol L}^{-1}$ ), and the culture was allowed to grow overnight at  $20^\circ\text{C}$ . Cells were harvested by centrifugation and resuspended in lysis buffer ( $20 \text{ mmol L}^{-1}$  Tris pH 7.4,  $0.2 \text{ mol L}^{-1}$  NaCl, 1:500 diluted protease inhibitor cocktail (Sigma-Aldrich), 50 Units Benzonase nuclease,  $0.1 \text{ mmol L}^{-1} \text{ ZnCl}_2$ ). Cells from 15 Liter 2YT were lysed using cell disruptor, clarified by centrifugation ( $20,000 \text{ rpm}$ ,  $4^\circ\text{C}$ , 30 min) and passed through a column packed with amylose beads (New England BioLab) pre-equilibrated with buffer A ( $20 \text{ mmol L}^{-1}$  Tris pH 7.4,  $0.2 \text{ mol L}^{-1}$  NaCl,  $0.1 \text{ mmol L}^{-1} \text{ ZnCl}_2$ ). Following an extensive wash with buffer B ( $20 \text{ mmol L}^{-1}$  Tris pH 8.0,  $0.2 \text{ mol L}^{-1}$  NaCl,  $0.1 \text{ mmol L}^{-1} \text{ ZnCl}_2$ ), the MBP-PTE fusion proteins were eluted with buffer B containing  $10 \text{ mmol L}^{-1}$  maltose and 10% glycerol. The fractions containing pure MBP-PTE were pooled and incubated with factor Xa at RT for 1 h followed by their incubation at  $4^\circ\text{C}$  with simultaneous dialysis over night with buffer B supplemented with 10% glycerol ( $20 \text{ mmol L}^{-1}$  Tris pH 8.0,  $200 \text{ mmol L}^{-1}$  NaCl and  $100 \mu\text{mol L}^{-1} \text{ ZnCl}_2$ ). The factor Xa digested

MBP-PTE was loaded on ion exchange column (Tricorn Q10/100GL). The flow through so obtained was concentrated and loaded on Hiload 26/60 Superdex 75 equilibrated with 50 mmol L<sup>-1</sup> Tris (pH 8.0) + 500 mmol L<sup>-1</sup> NaCl. The pure fractions of PTE were pooled and dialysed overnight with 50 mmol L<sup>-1</sup> Tris (pH 8.0) + 100 mmol L<sup>-1</sup> NaCl + 10 μmol L<sup>-1</sup> ZnCl<sub>2</sub>.

Protein concentrations were examined by absorbance at 280 nm (extinction coefficient value for the MBP fused enzymes was  $\epsilon = 95925$  OD/M).

### 2.3. In vitro efficiency of the PTE variant C23AL

The detoxification of VX by the PTE variant C23AL was investigated by using an AChE inhibition assay. C23AL (50 nmol L<sup>-1</sup>) was incubated with VX (6 μmol L<sup>-1</sup>). After defined time points, aliquots were transferred into cuvettes to measure AChE inhibition curves, which were quantified by the calculation of the first-order inhibition rate constant  $k_1$  (Aurbek et al., 2006). From these data, the first-order detoxification constants  $k_{obs}$  were calculated. With  $k_{obs}$  and the enzyme concentration [E], the ratio of  $k_{cat}$  to  $K_M$  was determined according to Eq. (1) (Dawson et al., 2008).

$$\frac{k_{cat}}{K_M} = \frac{k_{obs}}{[E]} \quad (1)$$

### 2.4. 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 animal protection committee (Ref.-No. 42-34-30-20/G03-15).

### 2.5. Experimental procedure

Terminally anesthetized guinea pigs were used (i.m. injection of a mixture of medetomidine (0.2 mg/kg), midazolam (1.0 mg/kg) and fentanyl (0.025 mg/kg) to reduce the suffering of the animals after VX challenge (subcutaneous injection of 18 μg kg<sup>-1</sup> VX) and to allow intraosseous (i.o.) injection. 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 positive foot withdrawal reflex or voluntary movements mostly around every 60 min. 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 (for protocol flow chart see Fig. 1). For the i.o.

application of the PTE a small incision was done and a 23 gauge needle (0.6 × 25 mm; Terumo, Eschborn, Germany) was carefully drilled by hand into the medullary cavity of the proximal tibia (see supplemental material). For intramuscular (i.m.) application in the left thigh a 30 gauge cannula (0.3 × 12 mm, Braun, Melsungen, Germany) was used.

### 2.6. Experimental groups

The animals were randomly divided into five groups:

- C23AL i.v. 1 mg kg<sup>-1</sup> (n = 3; group: C23AL i.v. control)
- C23AL i.o. 1 mg kg<sup>-1</sup> (n = 4; group: C23AL i.o. control)
- C23AL i.m. 1 mg kg<sup>-1</sup> (n = 2; group: C23AL i.m. control)
- C23AL i.v. 2 mg kg<sup>-1</sup> 5 min after 18 μg kg<sup>-1</sup> VX s.c. (n = 6; group: C23AL i.v.)
- C23AL i.o. 2 mg kg<sup>-1</sup> 5 min after 18 μg kg<sup>-1</sup> VX s.c. (n = 6; group: C23AL i.o.)

### 2.7. AChE assay

Multiple blood samples (250 μl, the volume was replaced by saline) were taken between 0 and 180 min with heparin as anticoagulant. 50 μl of whole blood were diluted 1:20 in ice-cold distilled water for the measurement of AChE activities (50 μl). The rest of the sample was centrifuged and separated into plasma and erythrocytes for C23AL and VX determination. All samples were shock-frozen in liquid nitrogen, and stored at -80 °C until measurement of AChE activity.

At the end of the experiment the brain was removed, immediately dissected into medulla, frontal cortex, striatum and hippocampus, shock frozen in liquid nitrogen and stored at -80 °C until analysis of AChE activity. Likewise, diaphragm was removed, shock frozen and stored at -80 °C.

Brain parts were mixed with a tenfold volume of phosphate buffer (0.1 mol L<sup>-1</sup>, pH 7.4) containing 1% Triton X-100. Diaphragms were mixed with a tenfold volume of TRIS buffer (50 mmol L<sup>-1</sup>, pH 7.4) supplemented with EDTA (5 mmol L<sup>-1</sup>), sodium chloride (1 mol L<sup>-1</sup>) and 1% Triton X-100. Both, the brain parts and diaphragms were subsequently homogenized in a glass-teflon Potter (Braun, Darmstadt, Germany) on ice. The homogenates were centrifuged (Hettich Microfuge 22, Tuttlingen, Germany) at maximum speed for 1 min and the supernatant was used for the AChE assay.

Brain, diaphragm and erythrocyte AChE activities were measured with a modified Ellman assay (Worek et al., 1999) at 436 nm (Cary 50, Varian, Darmstadt, Germany) using polystyrol cuvettes, 0.45 mmol L<sup>-1</sup> ATCh as substrate, 0.02 mmol L<sup>-1</sup> ethopropazine as selective BChE inhibitor and 0.3 mmol L<sup>-1</sup> DTNB as a chromogen in 0.1 mol L<sup>-1</sup> phosphate buffer (pH 7.4). Erythrocyte AChE activity was referred to the hemoglobin concentration of the

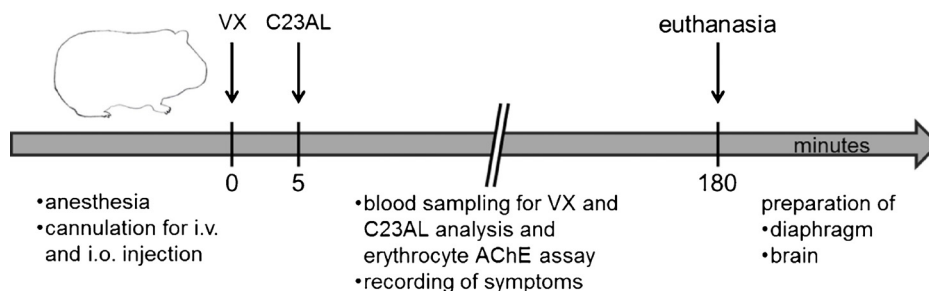


Fig. 1. Protocol flow chart.

individual blood dilution, determined with the cyanmethemoglobin method, and was expressed as mU/ $\mu\text{mol}$  Hb while brain and diaphragm AChE activity were calculated as mU/mg wet weight.

## 2.8. C23AL analysis

C23AL levels in plasma were determined by hydrolysis of 3-cyano-7-hydroxy-4-methylcoumarin (CMP) as previously described (Gupta et al., 2011; Worek et al., 2014b). Briefly, the initial velocity was monitored at 400 nm following 20  $\mu\text{l}$  plasma dilution in 1 ml 50 mmol L<sup>-1</sup> TRIS-HCl pH 8.0 plus 50 mmol L<sup>-1</sup> NaCl, containing 100  $\mu\text{mol}$  L<sup>-1</sup> CMP. A pre-determined calibration curve generated by adding increasing amounts of C23AL to equal volume of assay sample containing 20  $\mu\text{l}$  of naïve guinea pig plasma was used to calculate C23AL in plasma samples. Guinea pig plasma is devoid of CMP hydrolytic activity.

## 2.9. Analysis of VX in blood samples

The quantification of VX enantiomers was performed by LC-MS/MS according to Reiter et al. (2011, 2008) with several modifications. 100  $\mu\text{L}$  of thawed guinea pig plasma and erythrocytes, were diluted with 400  $\mu\text{L}$  of water and mixed with 37.5  $\mu\text{L}$  1 mol L<sup>-1</sup> perchloric acid followed by 7.5  $\mu\text{L}$  1 mol L<sup>-1</sup> potassium acetate. The precipitated proteins were separated by centrifugation for 5 min at 9000g and the supernatant was mixed with 50  $\mu\text{L}$  of 0.5% ammonia and 5  $\mu\text{L}$  of internal standard solution before it was transferred to a preconditioned (1 mL methanol, 1 mL water) SPE cartridge (Strata-X PRP, 30 mg, 1 mL, Phenomenex, Aschaffenburg, Germany). The sample was sucked through by moderate vacuum, the bed rinsed with 1 mL water and then thoroughly dried by maximum vacuum. For elution, 500  $\mu\text{L}$  acetonitrile was added to the cartridge, sucked through and collected in a cup containing 50  $\mu\text{L}$  of water as keeper. The organic phase was evaporated in a vacuum concentrator (60 min at room temperature) and the residue was reconstituted with 100  $\mu\text{L}$  water.

The analytical LC-MS/MS system for the quantification of VX enantiomers consisted of a Prominence 20A liquid chromatography system (Shimadzu, Neufahrn, Germany) and an API 6500 Q-Trap mass spectrometer (AB Sciex, Darmstadt, Germany).

Column: Chiralpak AGP column 150  $\times$  2.0 mm (Daicel, VWR, Ismaning, Germany)

Flow rate: 175  $\mu\text{L}$  min<sup>-1</sup>

Injection volume: 10  $\mu\text{L}$  at a column temperature of 30 °C

Mobile phases: A = 25 mM ammonium formate (pH 8.5) in water

B = 25 mM ammonium formate (pH 8.5) in 1:1 methanol-water

Linear gradients: Separation of the VX enantiomers was achieved by a 100% A (0–3 min), 100–95% A (3–3.1 min), 95–85% A (3.1–6 min), 85% A (6–18 min), 85–60% A (18–20 min), 60% A (20–32 min), 60–100% A (32–36 min), 100% A (36–40 min). During the first 20 min and again between 34 and 40 min, the mass spectrometer was flushed with a 10% methanol-water mixture by the make-up pump while the column effluent was directed to the waste by a column switching valve. Only during elution time of the VX enantiomers, the column was connected to the mass spectrometer.

## 2.10. Mass spectrometric detection

Two MRM transitions each for VX and VR (internal standard) were selected for detection after positive electrospray ionization at 5.5 kV with a source temperature of 400 °C and applying the following declustering potentials and collision energies, respectively:  $m/z$  268  $\rightarrow$  128 (120, 25 V),  $m/z$  268  $\rightarrow$  86 (146, 32 V) for VX and  $m/z$  268  $\rightarrow$  100 (120, 25 V),  $m/z$  268  $\rightarrow$  72 (121, 42 V) for VR. Gas

1, gas 2, and curtain gas were set at 35, 40, 45 arbitrary units, respectively, while entrance potential and cell exit potential were set at 5 and 12 V. Dwell time was 750 ms and resolution was “unit” for both quadrupoles.

## 2.11. Data analysis

Data are presented as mean  $\pm$  standard deviation (SD). Statistical comparisons were performed using GraphPad Prism Version 5.04 (GraphPad Software, San Diego, CA, USA). Differences of brain and diaphragm AChE activities between groups were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. A  $p < 0.05$  was considered to be statistically significant.

## 3. Results

### 3.1. In vitro activity of C23AL

PTE variant C23AL hydrolyzed the nerve agent VX with a  $k_{\text{cat}}/K_M$  value of  $1.2 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$  and was thus twice as active as the previously tested variant C23 with a  $k_{\text{cat}}/K_M$  value of  $0.6 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$  (Cherny et al., 2013; Goldsmith et al., 2015). Since C23AL was expressed as a maltose-binding-protein (MBP)-tagged fusion protein and then excised and purified from its MBP fusion tag, it had a substantially lower molecular weight of 36.9 kDa. Thus, in contrast to a previous *in vivo* experiment where the engineered PTE variant C23 was injected as a 79 kDa fusion protein (Worek et al., 2014b), untagged C23AL was injected as a protein having a 53% lower molecular weight. Since C23AL also had a 200% greater catalytic efficiency ( $k_{\text{cat}}/K_M$ ) of VX hydrolysis, we expected to obtain a fourfold higher activity of C23AL compared to C23 at any given dose.

### 3.2. In vivo plasma profile of C23AL after intramuscular, intravenous and intraosseous injection

For the control groups 1 mg kg<sup>-1</sup> was used as calculated minimal protective dose of the enzyme. Administration of 1 mg kg<sup>-1</sup> C23AL without VX challenge resulted in a plasma  $C_{\text{max}}$  of  $0.29 \pm 0.01 \mu\text{mol L}^{-1}$  3 min after i.v. injection and  $0.25 \pm 0.02 \mu\text{mol L}^{-1}$  after i.o. injection. At 180 min the level was  $0.10 \pm 0.01 \mu\text{mol L}^{-1}$  for i.o. and  $0.09 \pm 0.02 \mu\text{mol L}^{-1}$  for i.v. administration. The i.m. injection of 1 mg kg<sup>-1</sup> C23AL did not result in detectable plasma enzyme activities during 180 min in two guinea pigs (data not shown). The application of 1 mg kg<sup>-1</sup> C23AL i.v. and i.o. alone did not result in any clinical signs (data not shown). For the therapeutic groups 2 mg kg<sup>-1</sup> C23AL were applied by i.v. and i.o. injection and resulted in peak plasma concentrations of 0.63  $\mu\text{M}$  (Fig. 2). After

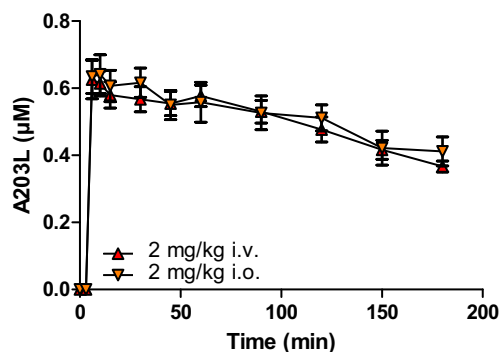


Fig. 2. C23AL plasma concentration in VX poisoned and C23AL treated guinea pigs. 18  $\mu\text{g kg}^{-1}$  VX s.c followed after 5 min by 2 mg kg<sup>-1</sup> C23AL i.v. or 2 mg kg<sup>-1</sup> C23AL i.o. Data are given in  $\mu\text{mol L}^{-1}$  as means  $\pm$  SD.

180 min the level decreased to 0.41  $\mu\text{M}$  with i.o. and 0.37  $\mu\text{M}$  with i.v. injection. Notably, doubling the dose of the C23AL increased as expected approximately 2-fold the enzyme plasma level at 3 min; however, at 180 min significant greater levels were retained ( $\approx 4$ -fold) compared to the dose of 1 mg/kg C23AL. It is not clear whether the presence of VX in the circulation is involved in keeping high plasma level compared to Guinea pigs treated with 1 mg/kg C23AL without VX challenge. Due to the limited observation period no assessment of the elimination kinetics was possible.

### 3.3. Effect of C23AL in VX poisoned guinea pigs

Subcutaneous injection of 18  $\mu\text{g kg}^{-1}$  ( $\sim 2\text{LD}_{50}$ ) VX (Wetherell et al., 2006; Worek et al., 2014b) resulted in rapid onset of cholinergic signs. Cholinergic crisis developed after 20–30 min (salivation, bronchorrhea, local convulsions, generalized convulsions, respiratory depression) and all animals died between 63 and 107 min after s.c. VX injection ( $85 \pm 13$  min) (data from (Worek et al., 2014b)). In the 2 mg  $\text{kg}^{-1}$  C23AL i.v. and i.o. therapy groups all animals challenged with 18  $\mu\text{g kg}^{-1}$  VX survived the observation period of 3 h (Fig. 3). Cholinergic intoxication symptoms developed later in comparison to untreated animals (c.f. (Worek et al., 2014b)) and were in part transient.

### 3.4. Erythrocyte AChE activity

The AChE activity levels of animals prior to intoxication and treatment were  $151 \pm 20 \text{ mU } \mu\text{mol}^{-1} \text{ Hb}$  for the i.v. treated group and  $147 \pm 7 \text{ mU } \mu\text{mol}^{-1} \text{ Hb}$  for the i.o. treated group. After VX poisoning AChE activity decreased rapidly in the absence of therapy showing almost complete inhibition after 6 min (Fig. 4). In both the i.v. and i.o. treatment group the decrease in erythrocyte AChE activity was comparable and resulted in an almost complete

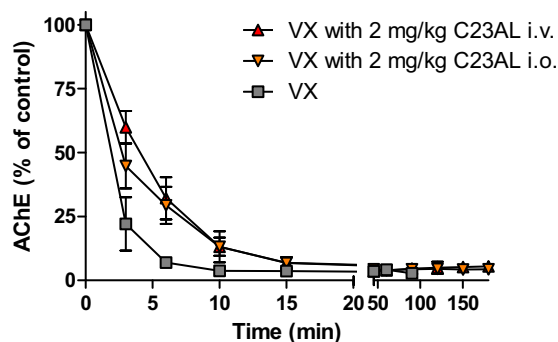


Fig. 4. Time-dependent changes of erythrocyte AChE activity. 18  $\mu\text{g kg}^{-1}$  VX s.c., taken from (VX, Worek et al., 2014b), 18  $\mu\text{g kg}^{-1}$  VX s.c. followed after 5 min by 2 mg  $\text{kg}^{-1}$  C23AL i.v. (VX with 2 mg/kg C23AL i.v.), 18  $\mu\text{g kg}^{-1}$  VX s.c. followed after 5 min by 2 mg  $\text{kg}^{-1}$  C23AL i.o. (VX with 2 mg/kg C23AL i.o.). Data are shown as % of saline control activity as means  $\pm$  SD.

inhibition after 15 min. The post-poisoning enzymatic treatment was not able to prevent erythrocyte AChE inhibition but was found to delay the decrease in enzyme activity. Erythrocyte AChE activities in the non-poisoned control groups were not affected by the i.v. or i.o. administration of 1 mg  $\text{kg}^{-1}$  C23AL (data not shown).

### 3.5. Brain AChE activity

VX poisoning resulted in complete inhibition of AChE activity in the medulla oblongata, cortex, striatum and hippocampus of untreated animals. Post intoxication treatment 5 min from intoxication, using a dose of 2 mg  $\text{kg}^{-1}$  C23AL resulted in partial protection of AChE activity in the brains of treated animals (Fig. 5). In the i.o. C23AL treatment group, AChE activity levels were

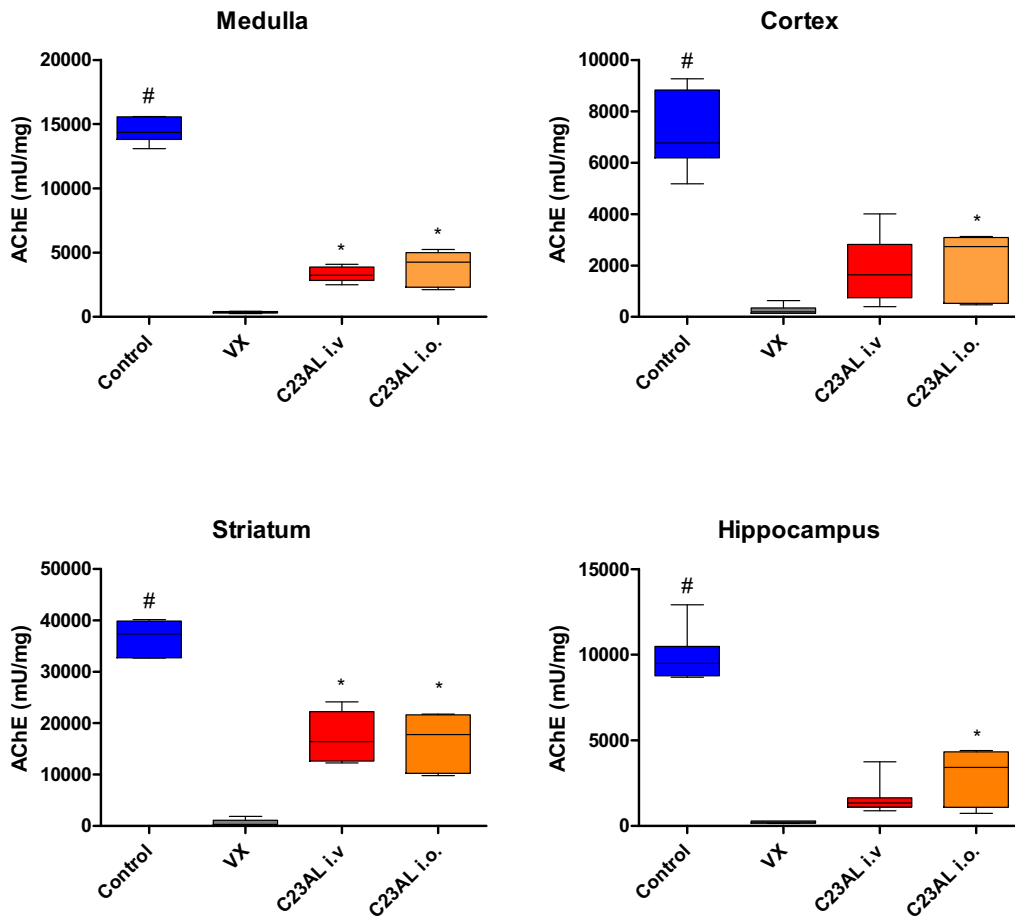
#### (A) 18 $\mu\text{g kg}^{-1}$ VX s.c. followed by 2 mg $\text{kg}^{-1}$ C23AL i.v. after 5 min

Signs	0 - 20	21 - 40	41 - 60	90	120	150	180
Salivation / Bronchorrhea							
Convulsions							
Altered respiration							
Respiratory depression							

#### (B) 18 $\mu\text{g kg}^{-1}$ VX s.c. followed by 2 mg $\text{kg}^{-1}$ C23AL i.o. after 5 min

Signs	0 - 20	21 - 40	41 - 60	90	120	150	180
Salivation / Bronchorrhea							
Convulsions							
Altered respiration							
Respiratory depression							

Fig. 3. Clinical signs and survival of VX poisoned guinea pigs treated with C23AL. Each line represents a single animal and the time is given in minutes. The signs are subdivided into salivation/bronchorrhea, convulsions (local or generalized), altered respiration (labored breathing) and respiratory depression (frequency, rhythm, gasping). (A) 18  $\mu\text{g kg}^{-1}$  VX s.c. followed after 5 min by 2 mg  $\text{kg}^{-1}$  C23AL i.v. and (B) 18  $\mu\text{g kg}^{-1}$  VX s.c. followed after 5 min by 2 mg  $\text{kg}^{-1}$  C23AL i.o.



**Fig. 5.** Brain AChE activity of saline control, VX and C23AL treated guinea pigs. Data of medulla, cortex, striatum and hippocampus AChE activity (mU/mg) are given as box plot with min to max whiskers. # versus VX, C23AL i.v. and C23AL i.o.;  $p < 0.05$ . \* versus VX group;  $p < 0.05$ . Data from saline control and VX groups were taken from (Worek et al., 2014b).

significantly higher in all tissues examined, while in the i.v. treatment group only the AChE levels in the medulla and striatum were greater than those of untreated animals. Yet, brain AChE activity in all tested brain areas, following the VX challenge and i.v. or i.o. PTE post-treatment was significantly lower in comparison with non-poisoned control animals.

### 3.6. Diaphragm AChE activity

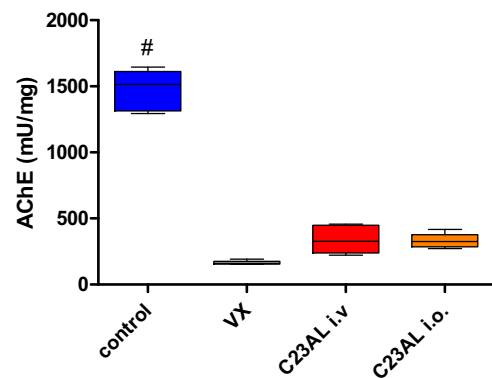
VX poisoning resulted in a marked inhibition of diaphragm AChE activity leading to ~10% residual AChE activity after death in untreated, VX intoxicated animals (Fig. 6). Treatment with  $2 \text{ mg kg}^{-1}$  C23AL 5 min post-intoxication resulted in a residual AChE activity of ~20% for both the i.v. and i.o. group. This substantial inhibition is in line with the clinical signs and explains the labored breathing and respiratory disturbance observed in some animals (Fig. 3).

### 3.7. VX analytics

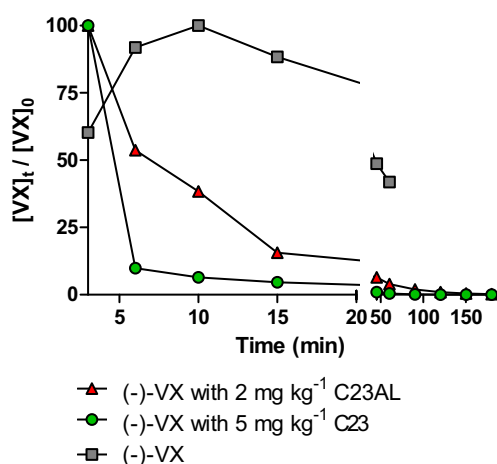
Analysis of the toxic (–)-VX enantiomer concentration in the plasma of VX poisoned and C23AL treated animals resulted in negligible concentrations while in erythrocytes peak concentrations of  $0.7$  and  $1.1 \text{ nmol L}^{-1}$  (–)-VX in the i.v. and i.o. group, respectively, were determined. Due to differences of VX analysis in the present and the previous study (Worek et al., 2014b) the

(–)-VX concentrations of i.v. C23- and C23AL- treated guinea pigs were transformed to% of the respective peak concentration.

The results revealed higher relative residual (–)-VX concentrations with  $2 \text{ mg kg}^{-1}$  C23AL i.v. compared to the previously tested C23 mutant (Fig. 7), most probably due to a slower degradation (Worek et al., 2014b). Non-linear regression analysis of relative (–)-VX concentrations revealed a one phase exponential



**Fig. 6.** Diaphragm AChE activity of saline control, VX and C23AL treated guinea pigs. Data of diaphragm AChE activity (mU/mg) are given as box plot with min to max whiskers. # versus VX, C23AL i.v. and C23AL i.o.;  $p < 0.05$ .



**Fig. 7.** Relative (–)-VX concentration in samples from guinea pigs poisoned by  $18 \mu\text{g kg}^{-1}$  VX s.c. alone and treated with  $2 \text{ mg kg}^{-1}$  C23AL i.v. (combined erythrocyte and plasma) and  $5 \text{ mg kg}^{-1}$  C23 i.v. (whole blood), respectively. The relative (–)-VX concentration was calculated as% of the highest determined concentration. Data are given as means. VX concentration data for VX alone and C23 were taken from (Worek et al., 2014b).

decay with a half-life for the (–)-VX of 1 min with  $5 \text{ mg kg}^{-1}$  C23 i.v. and 5 min with  $2 \text{ mg kg}^{-1}$  C23AL i.v. ( $R^2 > 0.99$ ). In consequence, detectable (–)-VX concentrations were present in the C23AL group for up to 90 min.

#### 4. Discussion

In this work we used a new PTE variant C23AL as a tool for the assessment of different administration routes of catalytic bioscavengers during post-intoxication treatments of VX intoxicated guinea pigs. In a recent study Worek et al. showed that the success of therapeutic i.v. injection of a PTE variant following VX exposure is time-dependent and should be administered as fast as possible after poisoning (Worek et al., 2014b). Yet, a successful application of an i.v. line in the field is time-consuming and necessitates skillful and experienced personnel. Thus, established alternative routes for a rapid and safe administration of enzyme-based drugs were tested in the current study. The i.m. injection was used to simulate an autoinjector as standard device for self and buddy aid in emergency treatment of nerve agent poisoning (Thiermann et al., 2013) and i.o. injection was tested to simulate the growing importance of intraosseous vascular access systems in emergency medicine (Neumar et al., 2010; Soar et al., 2015).

The i.m. injection did not result in a detectable plasma concentration of the injected PTE variant during the 180 min experimental period. This result is similar to results from experiments with human BChE, used as stoichiometric scavenger in guinea pigs, in which the  $C_{\text{max}}$  after i.m. injection was reached only after 26 h (Mumford et al., 2013). PTE appears mostly as a dimer in solution (unpublished results), thus even the untagged protein is estimated to circulate as a 73.4 kDa dimeric protein. The large molecular weight of the PTE dimer results in a slow diffusion velocity into the capillary system, which is a major determinant for the resorption of i.m. administered therapeutics. During a cholinergic crisis the blood circulation might be additionally impaired mitigating the resorption of the PTE. Mumford et al. injected human BChE intramuscularly (i.m.) after percutaneous (p.c.) VX exposure, which results in a slower rate of nerve agent transfer into systemic circulation. The resorption of p.c. VX is much slower than after s.c. injection and obviously allows a sufficient resorption of BChE from its i.m. injection site and the prevention of

cholinergic intoxication signs following p.c. VX exposure (Mumford et al., 2013; Mumford and Troyer, 2011). However, in the current study, a VX challenge by subcutaneous route had to be used due to the necessity to apply anesthesia, which limited the total duration of the experiment and mitigates convulsions—one of the recorded signs (Fig. 3). Taken together, an i.m. administration does not seem to provide an applicable route for C23AL, stand alone, post-intoxication treatment following a s.c. injection of VX.

Currently, the standard route of administration in experiments with catalytic (Gresham et al., 2010; Hodgins et al., 2013; Jackson et al., 2014; Valiyaveetil et al., 2011; Worek et al., 2014a, 2014b) and stoichiometric bioscavengers (Cerasoli et al., 2005; Huang et al., 2008; Mumford et al., 2013; Mumford and Troyer, 2011; Worek et al., 2014c) is the i.v. route. To the best of our knowledge, this is the first study that demonstrates the successful protection of VX poisoned animals by an i.o. injection of a catalytic bioscavenger. There seems to be no difference in the degree of protection of erythrocyte AChE (Fig. 4) and diaphragm AChE (Fig. 6) activities following the VX challenge between the i.o. and i.v. PTE administration routes. However, in some parts of the brain AChE activity was significantly better protected in i.o. treated animals compared to i.v. treated animals following VX intoxication (Fig. 5).

The maximal C23AL plasma concentration following the introduction of a  $2 \text{ mg kg}^{-1}$  C23AL dose in both the i.o. and i.v. routes was  $0.63 \mu\text{mol L}^{-1}$  after 1 min (Fig. 3). Assuming  $39 \text{ ml/kg}$  plasma in guinea pigs, the expected initial concentration of C23AL was  $51.0 \mu\text{g/ml}$ . The observed value of  $23.2 \mu\text{g/ml}$  ( $0.63 \mu\text{mol L}^{-1} \times 36.9 \text{ mg/1000}$ ), suggests that the PTE was distributed in approximately 2-fold larger volume than the plasma, compared to  $44.3 \mu\text{g/ml}$  ( $0.56 \mu\text{mol L}^{-1} \times 79.2 \text{ mg/1000}$ ) in the case of C23 that seems to distribute in the plasma content of the animal. Thus, the volume of distribution of C23AL is approximately twice the volume of distribution of C23, an observation that may be attributed to the larger size of the C23 variant. These plasma concentrations were reduced to  $0.41 \mu\text{mol L}^{-1}$  and  $0.37 \mu\text{mol L}^{-1}$  in the i.o. and i.v. groups respectively after 180 min. While the peak plasma concentrations of C23AL were similar to that obtained in a previous study (Worek et al., 2014b) using the same  $2 \text{ mg kg}^{-1}$  dose of an MBP-tagged PTE variant C23 ( $0.56 \mu\text{mol L}^{-1}$ ), the 3 h plasma concentrations of C23AL were much higher than obtained with C23 ( $0.13 \mu\text{mol L}^{-1}$ ) (Worek et al., 2014b). This may indicate that while the maltose-binding-protein tag is beneficial for *in vitro* protein stability of PTE variants, it may increase their clearance from plasma *in vivo*.

Knowledge of the *in vitro* catalytic activity of C23AL, i.e.  $k_{\text{cat}}/K_M$  of  $1.2 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ , and its plasma concentration allows the calculation of the theoretical hydrolysis half-life of the toxic VX(–) enantiomer. Hence, the determined peak plasma level that appeared to be unchanged over the next 10 min, should result in a calculated half-life of 5.39 s [ $t_{1/2} = 0.69 / (1.2 \times 10^7 \times 0.63 \times 10^{-6})$ ]. Consequently, the degradation of 96.75% (–)-VX, i.e. five half-lives, should theoretically be achieved within  $\approx 27$  s. This would be even faster than the calculated 36 s with  $5 \text{ mg kg}^{-1}$  C23 with an identical VX challenge (Worek et al., 2014b). However, the expected degradation rate of VX was not obtained in the presented *in vivo* model. In contrast, the activity obtained was lower in comparison to C23 *in vivo* (Fig. 7). The more severe clinical signs and symptoms (Fig. 3) compared to the ones found in the C23 study are in line with a slower VX detoxification (Worek et al., 2014b). One explanation for the gap between the theoretical calculations and *in vivo* data might be an adsorption or unspecific binding of the PTE mutant C23AL to plasma proteins that increase the volume of distribution resulting in a lower activity in the body. However, this would not be reflected by low or varying C23AL plasma concentrations since in the applied assay plasma samples are highly diluted *ex vivo* which could then result in a release of the

loosely bound C23AL. In the end, regardless of the data interpretation, these results underline the necessity for additional *in vivo* experiments in the further development of catalytic bioscavengers.

In conclusion, the present study demonstrates for the first time the suitability of intraosseous injection for the administration of a PTE bioscavenger during post-intoxication treatment of nerve agent poisoned guinea pigs. The intraosseous injection resulted in PTE plasma levels comparable to an i.v. administration and resulted in a similar therapeutic efficacy. I.o. vascular access systems may allow a safe and rapid administration of a catalytic bioscavenger to the systemic circulation and could improve the post-exposure PTE therapy of nerve agent poisoning.

### Conflict of interest

None.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.toxlet.2016.07.004>.

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