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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_{50}).

C23AL showed a C_{max} of 0.63 μmol L^{-1} after i.o. and i.v. administration of 2 mg kg^{-1} providing a stable plasma profile up to 180 min experimental duration with 0.41 and 0.37 μmol L^{-1} 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_{cat}/K_M values > 10^7 $M^{-1}min^{-1}$– a bench mark for effective detoxification at reasonable enzyme doses (<1 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_{cat}/K_M values of up to 5×10^6 M^{-1} 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 $2LD_{50}$ of VX (Worek et al., 2014b). From this promising starting point a further mutant with a higher activity *in vitro* (k_{cat}/K_M of 1.2×10^7 M^{-1} min^{-1} vs 0.6×10^7 M^{-1} 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 (≤2 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 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}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) 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 °C. The inoculate was dilute 1:100 into LB medium with ampicillin (100 μg/ml) and 0.2 mmol L^{-1} $ZnCl_2$ and grown at 37 °C to $OD_{600nm} \approx 0.6$. IPTG was added (0.4 mmol L^{-1}), and the culture was allowed to grow overnight at 20 °C. Cells were harvested by centrifugation and re-suspended in Lysis buffer (20 mmol L^{-1} Tris pH 7.4, 0.2 mol L^{-1} NaCl, 1:500 diluted protease inhibitor cocktail (Sigma-Aldrich), 50 Units Benzonase nuclease, 0.1 mmol L^{-1} $ZnCl_2$). Cells from 15 Liter 2YT were lysed using cell disruptor, clarified by centrifugation (20,000 rpm, 4 °C, 30 min) and passed through a column packed with amylose beads (New England BioLab) pre-equilibrated with buffer A (20 mmol L^{-1} Tris pH 7.4, 0.2 mol L^{-1} NaCl, 0.1 mmol L^{-1} $ZnCl_2$). Following an extensive wash with buffer B (20 mmol L^{-1} Tris pH 8.0, 0.2 mol L^{-1} NaCl, 0.1 mmol L^{-1} $ZnCl_2$), the MBP–PTE fusion proteins were eluted with buffer B containing 10 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 °C with simultaneous dialysis over night with buffer B supplemented with 10% glycerol (20 mmol L^{-1} Tris pH 8.0, 200 mmol L^{-1} NaCl and 100 μmol L^{-1} $ZnCl_2$). The factor Xa digested