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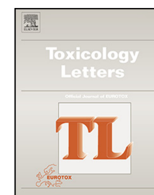

Post-exposure treatment of VX poisoned guinea pigs with the engineered phosphotriesterase mutant C23: A proof-of-concept study

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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 (~$2LD_{50}$) 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.

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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 $\sim$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 $\approx$ 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.

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