Research article

Drug Testing and Analysis

Received: 16 July 2011 Revised: 19 August 2011 Accepted: 22 August 2011 Published online in Wiley Online Library: 15 December 2011

(wileyonlinelibrary.com) DOI 10.1002/dta.363

In vitro and *in vivo* efficacy of PEGylated diisopropyl fluorophosphatase (DFPase)

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Highly toxic organophosphorus compounds that irreversibly inhibit the enzyme acetycholinesterase (AChE), including nerve agents like tabun, sarin, or soman, still pose a credible threat to civilian populations and military personnel. New therapeutics that can be used as a pretreatment or after poisoning with these compounds, complementing existing treatment schemes such as the use of atropine and AChE reactivating oximes, are currently the subject of intense research. A prominent role among potential candidates is taken by enzymes that can detoxify nerve agents by hydrolysis. Diisopropyl fluorophosphatase (DFPase) from the squid *Loligo vulgaris* is known to effectively hydrolyze DFP and the range of G-type nerve agents including sarin and soman. In the present work, DFPase was PEGylated to increase biological half-life, and to lower or avoid an immunogenic reaction and proteolytic digest. Addition of linear polyethylene glycol (PEG) chains was achieved using mPEG-NHS esters and conjugates were characterized by electrospray ionization – time of flight – mass specrometry (ESI-ToF-MS). PEGylated wildtype DFPase and a mutant selective for the more toxic stereoisomers of the agents were tested *in vivo* with rats that were challenged with a subcutaneous 3x LD_{50} dose of soman. While wildtype DFPase prevented death only at extremely high doses, the mutant was able keep the animals alive and to minimize or totally avoid symptoms of poisoning. The results serve as a proof of principle that engineered variants of DFPase are potential candidates for *in vivo* use if substrate affinity can be improved or the turnover rate enhanced to lower the required enzyme dose. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: PEGylation; bioscavenger; nerve agents; enzymes; ESI-TOF-MS

Introduction

Organophosphorus (OP) nerve agents including tabun (GA), sarin (GB), soman (GD, Figure 1), cyclosarin (GF), and VX pose a continuing threat to military personnel and civilian populations despite being banned under the Chemical Weapons Convention (CWC).[1] The main target of these OP compounds is the enzyme acetylcholinesterase (AChE), where an active site serine residue is phosphylated (phosphorylated or phosphonylated) rendering the enzyme inactive and therefore leading to the accumulation of acetylcholine and subsequent cholinergic crisis. [2] Treatment of intoxication with these agents is currently based on the use of atropine as a symptomatic antagonist of effects at muscarinic receptors,[3] oximes to reactivate inhibited AChE[4] and benzodiazepines to stop seizures and prevent seizure-related brain damage.[5]

With respect to the use of oximes as AChE reactivators, problems exist in particular with the nerve agents tabun and soman. Due to the phosphoroamidate structure in tabun, AChE inhibited by this agent is hard to reactivate by currently fielded oximes like obidoxime or 2-PAM.[6] In the case of soman, the main problem is the rapid 'aging' of the phosphylated AChE. Aging denotes the loss of an O-alkyl sidechain from the phosphylated moiety resulting in a negative charge at this group hindering the nucleophilic attack of a reactive oximate anion.[7] This aging reaction is especially rapid in case of soman with *in vivo* reaction half-lives of only a few minutes.[8] It should also be noted that oximes react with OP-inhibited AChE or the free agent to form phosphylated oximes that are also highly toxic.[9]

A new alternative approach focuses on the administration of enzymes or proteins that react with the organophosphorus compounds either stoichiometrically or catalytically. While these bioscavengers are preferably administered as prophylaxis prior to intoxication, they are also useful post-exposure as they clear the body of remaining agent that could re-inhibit AChE, which has already been reactivated during initial oxime therapy.[10] A stoichiometric bioscavenger currently in advanced development is human butyrylcholinesterase (BChE) and candidates

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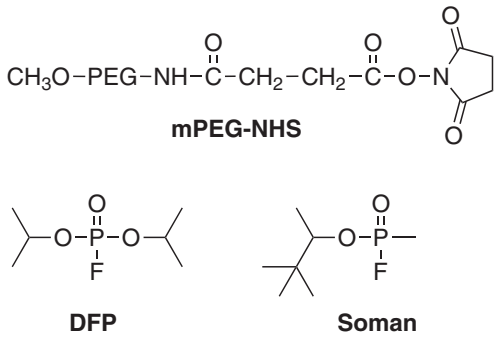

Figure 1. Structure of the reactive mPEG-NHS esters and of the organophosphorus compounds DFP and soman used in this study.

for catalytic bioscavengers include Paraoxonase (PON1) and bacterial organophosphorus hydrolase (OPH).[11–13]

Problems associated with non-human enzymes are rapid clearance from the body and immunogenicity. One solution to overcome both problems is the covalent linking of polyethylene glycol (PEG) chains to the protein.[14] This process also known as PEGylation was recently carried out with OPH.[15,16] PEGylation increases the hydrodynamic radius of proteins and shields its surface. This increases the stability against proteases, immunogenicity is reduced, and renal excretion is significantly delayed, leading to prolonged half-life, reduced side effects, and increased pharmacological efficiency.[17]

One enzyme that was developed for external decontamination of material and personnel over the past years is the well-characterized calcium-containing phosphotriesterase diisopropyl fluorophosphatase (DFPase, EC 3.1.8.2, 35 kDa) from the squid *Loligo vulgaris*,[18–20] which displays significant hydrolytic activity against diisopropyl fluorophosphate (DFP, Figure 1) and the range of G-type nerve agents including GA, GB, GD, and GF but is inactive against the range of V-type nerve agents including VX. The enzyme was structurally characterized by X-ray and neutron diffraction.[21–23] Recent enzyme engineering efforts yielded a DFPase variant with a preference for the more toxic enantiomers of the nerve agents (where the phosphorus atom forms a stereocenter) and enhanced enzymatic activity.[24] The reaction mechanism of DFPase is thought to proceed via coordination of the substrate phosphoryl oxygen to the catalytic calcium ion in the active site; the subsequent nucleophilic attack of residue D229 on the substrate phosphorus atom forms a phosphoenzyme intermediate, which is subsequently hydrolyzed to liberate the reaction product and regenerate the enzyme.[19]

In this work we report on the successful PEGylation of DFPase using linear PEGs with 750 Da and 2000 Da molecular weight and the characterization of the DFPase-PEG conjugates using ESI-MS techniques and kinetic *in vitro* experiments. We use PEGylated DFPase WT and mutant E37A/Y144A/R146A/T195M to demonstrate the *in vivo* efficacy of the enzyme to protect rats against a 3 x LD_{50} challenge of soman also proving the superior properties of the DFPase mutant favoring the more toxic enantiomers of soman compared to the WT.

Experimental

Chemicals and reagents

Methyl-PEG-*N*-hydroxysuccinimide (mPEG-NHS) esters (Figure 1) with PEG molecular weights of 750 and 2000 Da were purchased from Rapp Polymere (Tübingen, Germany). Anhydrous Acetonitrile for DFP stock solutions was from Sigma Aldrich (Taufkirchen, Germany). All other chemicals used were of analytical grade. Vivaspin concentrators (MWCO 10 kDa) were from Sartorius (Göttingen, Germany) and dialysis tubing (MWCO 12–14 kDa) was purchased from SERVA Electrophoresis (Heidelberg, Germany). *E. coli* BL21 competent cells were from Agilent/Stratagene (Waldbronn, Germany) and components of terrific broth (TB) media (peptone, yeast extract, glycerol), ampicillin and Isopropyl-β-D-thiogalactopyranosid (IPTG) were from Roth (Karlsruhe, Germany). Goat anti-rat IgG (Fc) was from Bethyl Laboratories Inc. (Montgomery, TX, USA) and biotinylated polyclonal rabbit anti-goat immunoglobulins, streptavidin/FITC, protein blocking solution and antibody diluent were from Dako (Hamburg, Germany). Maxisorp Immuno plates were obtained from Nunc (Langenselbold, Germany). Soman (*O*-pinacolyl methylphosphonofluoridate) was provided by the French single small scale facility (DGA maîtrise NRBC, Vert-le-Petit, France).

Animals

Male Wistar rats used in this study were obtained from Janvier-CERJ (Le Genest Saint Isle, France) weighing 250 to 274 g upon arrival. The animals were housed on a 12 h dark/light cycle with light provided between 7 am and 7 pm. They were given food and water *ad libitum*. Animals were treated according to the *Health guidelines for the care and use of laboratory animals* at CRSSA (Centre de Recherches du Service de Santé des Armées, now: Institut de Recherche Biomédicale des Armées - CRSSA, Département de Toxicologie et Risques Chimiques, La Tronche, France). Animal tests were approved and monitored by the Institutional Animal Care and Research Advisory Committee in accordance with the applicable French and European community regulations.

Expression of recombinant DFPase

WT DFPase and mutant E37A/R144A/Y146A/T195M were expressed in *E. coli* BL21 cells and purified according to the method of Hartleib and Rueterjans.[17] In short, cells were grown in TB-media containing 100 μg/ml of ampicillin and incubated at 37 °C. Cells were induced by addition of IPTG at $OD_{600} = 0.7$ to a final concentration of 1 mM. At the same time another batch of ampicillin (100 μg/ml) was added and the temperature lowered to 30 °C. After overnight incubation (12–16 h) the cells were harvested by centrifugation (30 min, 4 °C, 12 000 g). DFPase was purified by nickel nitrilo tri acetic acid (Ni-NTA) affinity chromatography and Q-Sepharose anion-exchange chromatography. After a first chromatographic step using Ni-NTA resin, the His-tagged DFPase was collected and the tag cleaved by digestion with thrombin. Re-chromatography on Ni-NTA yielded the DFPase without the tag. Final purification was achieved by the use of Q-Sepharose. The purified DFPase was concentrated and de-salted using a Vivaspin concentrator (10 kDa cutoff).

PEGylation of DFPase

PEGylations were carried out at different ratios of mPEG-NHS and enzyme. DFPase features 23 surface-exposed lysine residues, which in principle can react with PEG-NHS esters. mPEG-NHS used in 100% concentration therefore means that mPEG-NHS was used in a molar ratio of 23:1 so that all lysine residues can

be PEGylated theoretically. mPEG-NHS was used in concentrations of 10%, 50%, 200%, 500%. To 5 mg of DFPase (0.142 μmol) in 10 mM Tris buffer (2 mM $CaCl_2$, pH 7.5), the respective amount of the mPEG-NHS ester was added. The solution was diluted to a final volume of 1 ml using the same Tris buffer as above. The reaction mixture was incubated for 2 h at room temperature (RT). After this time surplus PEG was removed by ultrafiltration using a Vivaspin concentrator with an MWCO of 10 kDa. To the reaction solution 5 ml of 10 mM ammonium acetate solution (pH 7.0) were added and the solution concentrated to 1 ml using the concentrator. This procedure was repeated ten times. PEGylated DFPase was kept in ammonium acetate for analysis by MS and SDS-PAGE. For *in vitro* activity assays and *in vivo* work the buffer was exchanged to 10 mM HEPES (1 mM $CaCl_2$, 150 mM NaCl, pH 7.4).

SDS-PAGE was carried out using 12% Mini-PROTEAN precast gels (Biorad, Munich, Germany). Protein samples were mixed with 5x sampling buffer (250 mM Tris/HCl, 25% (*v/v*) glycerol, 12.4% (*v/v*) β-mercaptoethanol, 7.5% (*w/v*) SDS, 0.25 mg/ml bromophenol blue, pH 8.0) and heated for 10 min at 95 °C. After loading of the samples on the gel they were focused for 15 min at 100 V and separated at 200 V for 45 min. Afterwards the gels were stained in coomassie blue solution (80 mg/l coomassie brilliant blue G-250, 35 mM HCl in water) by heating in a microwave oven for 30 s and subsequent incubation at RT for 15–20 min. For destaining, the gel was incubated in water overnight.

ESI-ToF-MS detection of PEGylated DFPase

Mass spectrometric detection of PEGylated DFPase species was achieved by ESI-ToF-MS employing an Agilent 6210 TOF LC/MS system in positive ionization mode. Data recording was carried out using the Agilent MassHunter Workstation software A02.02 (2003–2005) while spectra processing and deconvolution was done by the Agilent MassHunter Workstation software version B.0102 (2006–2007). The sample of PEGylated DFPase was injected using direct infusion with a Harvard syringe pump (11 plus) at a flow rate of 9 μl/min equipped with a 100-μl Hamilton syringe. The Agilent 6210 TOF mass spectrometer was run under the following conditions: dry gasflow: 480 l/h; drying gas temperature: 300 °C; nebulizer gas pressure: 15 psig; scan mass range: *m/z* 150–*m/z* 3200; fragmentor voltage: 150 V; skimmer voltage: 60 V and capillary voltage: 3000 V. Deconvolution settings were applied as follows: deconvolution area: 34–60 kDa; mass step: 1; s/n: 30.

Determination of kinetic constants of PEGylated DFPase

Initial estimates of enzyme activity were carried out following the method of Gäb *et al*. using *in-situ* FTIR spectroscopy.[25] To determine the kinetic parameters K_M and k_{cat}, initial velocities were recorded using pH-Stat titration with DFP as substrate in the range from 10.5 mM to 1.75 mM using a Metrohm 799 GPT Titrino (Herisau, Switzerland). All measurements were carried out at 25 °C (298 K). The total volume was 3.0 ml, at pH 7.5, containing 10 mM NaCl and 10% acetonitrile, and the reaction was initiated by addition of 2 μl of 0.5 mg/ml DFPase (28.57 pmol). Initial velocities were corrected by the uncatalyzed rate of DFP hydrolysis. The kinetic parameters K_M and k_{cat} were determined by nonlinear least-squares fitting of the data to the Michaelis-Menten equation using the software GraphPad Prism 4.0. The parameters described are the average of at least three independent measurements.

Soman challenge, DFPase administration and monitoring of rats

On the day of the experiment, the rats used had an average weight of 345 g. Blood samples were collected from the retro-orbital sinus of the animals prior to the administration of enzyme (5 min prior to administration of soman) and after the soman challenge. Blood sampling was done under halothane anesthesia and samples (100 μl) were mixed with 3 μl sodium heparin, diluted 1:10 with sterile water and deep-frozen for the determination of whole blood (WB) cholinesterase (ChE) activity. Injection of enzyme solution or buffer solution for the control group (see Table 2 for enzyme doses used) was done directly in one of the tail veins after the first blood sampling. All solutions were sterile filtered before use. Five minutes later rats were challenged with approx. 3 x LD_{50} of soman (270 μg/kg) administered in a volume of 500 μl subcutaneously (*s.c.)* in the shaven side of the animals. WB ChE activity was determined using the Ellman assay (0.1 M phosphate buffer pH 8, 37 °C) with acetylthiocholine as a substrate (0.99 mM final concentration) and Ellman's reagent 5,5′-Dithio-bis(2-nitrobenzoic acid), DTNB (0.33 mM final concentration). WB ChE activity was determined in animals that survived soman challenge: 90 min after challenge for the PEGylated DFPase and ca. 3 h after challenge for the PEGylated DFPase mutant. Post-mortem blood sampling was also performed in some of the animals that did not survive the challenge. Percentage of inhibition was calculated from the activity determined before soman challenge. After the second blood sampling, surviving animals were monitored and weighed daily for seven days.

Determination of anti-DFPase antibodies in rat plasma by ELISA

Rat plasma for antibody detection was obtained from surviving rats that were treated with PEGylated DFPase mutant E37A/Y144A/R146A/T195M. Plasma samples were collected 14 and 27 days after enzyme treatment. One rat was injected a second time with a lower dose of enzyme (ca. 19 mg/kg), at day 17, three days after the first plasma was taken.

A Maxisorp Immuno 96-well plate was coated with the DFPase mutant in PBS buffer at three different concentrations (10 μg/ml, 50 μg/ml and 100 μg/ml in a volume of 900 μl) at 4 °C over night. The control was only treated with PBS buffer. Then the plate wells were washed three times with 0.005% Tween20 in PBS buffer (pH 7.4) and protein block solution (commercial product containing 0.25% casein in PBS with stabilizing protein and 0.015 mol/l sodium azide) was applied for 1 h at RT. After this the wells were washed again three times. Washing solution was removed and 25 μl of plasma sample were incubated in the well at 4 °C over night (PBS buffer in case of the control). The next day the wells were washed again and the primary antibody (goat anti-rat IgG Fc; 1 mg/ml) diluted 1:1000 in antibody diluent (commercial product containing Tris-HCl buffer with stabilizing protein and 0.015 mol/l sodium azide) was incubated in the wells for 2 h at RT. Wells were washed again to remove the primary antibody and the secondary antibody (biotinylated polyclonal rabbit anti-goat immunoglobulins; 1.3 mg/ml) diluted 1:500 in antibody diluent was applied and incubated for 2 h at RT. After another washing step streptavidin/fluorescein isothiocyanat (0.4 mg/ml) diluted 1:200 was applied and incubated for 20 min. Then the well plate was inserted into a LI-COR Odyssey infrared imaging system (LI-COR, Lincoln, NE, USA). An excitation wavelength of 488 nm was used and fluorescence emission was detected at 530 nm.

Results and discussions

PEGylation of DFPase and *in vitro* efficacy of PEG conjugates

PEGylation of therapeutic proteins has been applied to great extend to enhance overall effectiveness by prolonging circulation time in the body, increase resistance against proteases, reduce immunogenicity and lower cytotoxicity.[26–28] PEGylated proteins are not niche products. Some PEGylated protein drugs like PEGylated interferon, granulocyte colony-stimulating factor or epoetin-β even are 'blockbuster' drugs.[14]

DFPase from *Loligo vulgaris* was subjected to PEGylation studies before. In order to study the structural mechanism of the thermoinactivation of DFPase immobilized in polyurethane foam, PEG-modified DFPase was used as a model.[29] In this study, DFPase was linked with PEG-monoisocyanate (PEG-NCO) that reacts with free amino groups of the protein (lysine residues). Even though DFPase displays 23 surface-exposed lysine residues only up to eight PEG-chains were covalently bound to the protein. In the present study, a different and more widely used linker group was employed using an NHS-activated ester (Figure 1) that reacts with protein amino groups under very mild conditions, low temperatures and mild pH. With 23 surface-exposed lysine residues, DFPase offers a large number of potential reaction centers. One potential problem that can become relevant during PEGylation is that a residue in close proximity to or even in the active site of the enzyme could react with PEG and block access of substrate molecules and therefore significantly lower the enzyme activity.[30] Even though the 23 lysine residues are spread over the DFPase molecular surface, there is only one lysine (K269) found in the surroundings of the active site and none in the active site itself (Figure 2). K269 is 3.83 Å away from residue W244 that forms part of the active site-binding pocket and 14.19 Å away from the catalytic calcium ion. Strategies to avoid this problem would be mutation of the lysine residue or protection of the active site during PEGylation using a tight binding inhibitor or substrate.[31] We opted to PEGylate DFPase WT without modifications and test enzyme activity of the PEG-conjugates. We used two different mPEG-NHS reagents (with a PEG molecular mass of 750 and 2000 Da) and employed four different concentrations of the reagents (10%, 50%, 200%, and 500%); 100% PEG means that theoretically all 23 lysine residues can be PEGylated once (a molar protein:PEG ratio of 1:23).

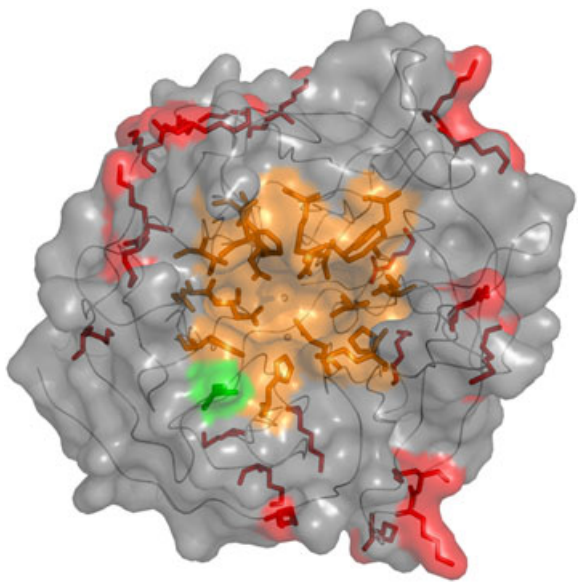

Figure 2. Representation of WT DFPase depicting the solvent accessible surface. The enzyme's active site can be seen in the center and is coloured orange. Lysine residues are shown in red and the lysine residue K269, which is closest to the active site, is marked in green.

Reaction mixtures were removed from unreacted PEG by ultrafiltration (MWCO 10000) because the use of dialysis tubing with the same cut-off turned out to be insufficient. Initial estimates of enzyme activity with DFP as the substrate to assess the required protein concentration and substrate range required for the determination of kinetic constants were obtained by a previously described method using *in situ* FTIR spectroscopy.[25] Recording of initial reaction rates over a range of DFP concentrations was then achieved using pH-stat titration. Kinetic constants k_{cat}, K_M, and k_{cat}/K_M for the DFPase conjugates resulting from different PEG reagent and different reaction compositions can be found in Table 1. As can be seen the k_{cat} and K_M values of the DFPase PEG conjugates reacted with 50% and 200% PEG-750 and 50% PEG-2000 do no differ much from the values of the native enzyme. A slight drop in k_{cat} and increase in K_M can be observed for reactions carried out with 10% PEG and very significant drop in enzyme efficiency again manifested in a lowered k_{cat} and a higher K_M are found for reactions with 500% PEG and also with 200% PEG in case of PEG-2000. From this data, reaction of DFPase with PEGs in a molar ratio of 1:11.5 (50% conditions) seems optimal to maintain enzyme activity but it remains unknown how many PEG chains are attached to the enzyme under these conditions. A first assessment was possible by analyzing an SDS-PAGE run with native DFPase and the different reaction products (Figure 3). It shows several bands for different conjugates of different molecular weight. Separation of bands is more pronounced in case of the conjugates with PEG-2000 and shows about 4–5 product bands with the exception of the products that resulted from 500% reaction conditions that only reveal a smear. This is indicative of unspecific enzyme-PEG complexes and for the fact that removal of excess PEG was unsuccessful. Unfortunately it is not possible to deduce the molecular weight of the conjugates by the position of the band relative to a molecular weight ladder because for PEGs and PEG-protein conjugates there is no correlation with protein molecular weight standards.[32]

Table 1. Kinetic parameters of DFPase hydrolysis determined by the pH-Stat method with DFP as substrate. Concentrations between 1.75 and 10.5 mM were used. The total initial volume was 2.5 ml at pH 7.5 with 100 mM NaCl and 10% acetonitrile. Initial velocities were corrected by the aqueous hydrolysis of DFP.

	k_{cat} [s^{-1}]	K_M [mM]	k_{cat}/K_M [$M^{-1}\,s^{-1}$]
PEGylated WT			
PEG750 10%	183.2 ± 6	3.86 ± 0.12	$4.8 \cdot 10^4$
PEG750 50%	214.5 ± 9	3.29 ± 0.11	$6.5 \cdot 10^4$
PEG750 200%	203.4 ± 6	3.27 ± 0.17	$6.2 \cdot 10^4$
PEG750 500%	29.4 ± 4	5.38 ± 0.24	$5.5 \cdot 10^3$
PEG2000 10%	194.9 ± 3	3.48 ± 0.11	$5.6 \cdot 10^4$
PEG2000 50%	229.2 ± 7	3.22 ± 0.03	$7.1 \cdot 10^4$
PEG2000 200%	195.1 ± 14	6.02 ± 0.17	$3.2 \cdot 10^4$
PEG2000 500%	33.9 ± 2	5.82 ± 0.25	$5.5 \cdot 10^3$
Native WT	219.7 ± 9	2.90 ± 0.05	$7.6 \cdot 10^4$
PEGylated Mut.			
PEG2000 50%	361 ± 9	4.12 ± 0.12	$8.7 \cdot 10^4$
Native Mut.	376.0 ± 13	3.93 ± 0.14	$9.6 \cdot 10^4$

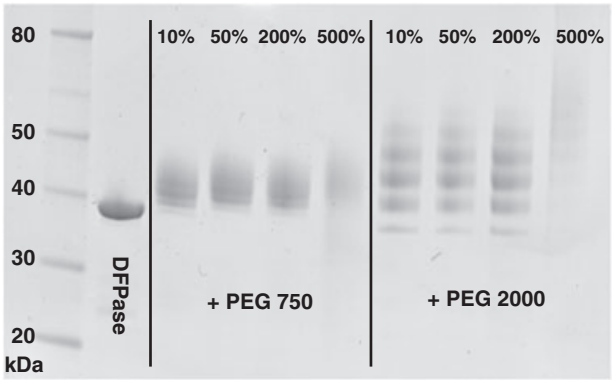

Figure 3. SDS-PAGE showing bands of native WT DFPase and reaction products with different ratios of mPEG-NHS (750 Da and 2000 Da). DFPase-PEG conjugates with different extent of PEGylation can clearly be identified by individual bands, especially in case of mPEG-NHS 2000 Da. Reaction conditions employing 500% PEG only yield a smear on the gel indicative of unspecific PEG-protein complexes.

Mass spectrometric characterization of PEGylated DFPase

Mass spectrometric characterization of PEGylated DFPase was carried out using direct infusion ESI-ToF-MS. First experiments with reaction solutions purified from unreacted PEGs by the use of dialysis led to overloaded spectra. A deconvolution of these spectra turned out to be impossible. This was due to the fact that dialysis did not allow the removal of PEG and therefore a complicated mixture of protein, protein-PEG conjugates and unreacted PEG was present in solution. After the purification strategy was changed to ultrafiltration using Vivaspin concentrators, clean spectra were recorded that allowed determination of the extent of PEGylation of the enzyme.

The recorded mass spectrum of native WT DFPase is shown in Figure 4a and the corresponding deconvoluted spectrum in Figure 4b. The DFPase used in these experiments was expressed with a N-terminal polyhistidine-tag and a thrombin cleavage site. In addition to the 314 amino acids of native DFPase two additional N-terminal residues (Gly-Ser) are present from this cleavage site. The resulting 316 residue protein has an average mass of 35224.48 Da (calculated: 35224.52 Da). The second highest peak with the mass of 35077.29 Da can be assigned to the protein with a missing C-terminal phenylalanine residue. Slow proteolytic digestion of the C-terminal residues over time was observed previously (unpublished data).

At 10% mPEG-NHS 750 Da only minor formation of PEGylated species can be observed. At 50% conditions reacts with DFPase so that almost no native protein remains in the reaction mixture. The dominant species formed have 2–3 PEG chains attached. The maximum number of PEG chains grafted to the protein is 4 with very minor peaks for conjugates with 5–6 PEG chains. At 200%

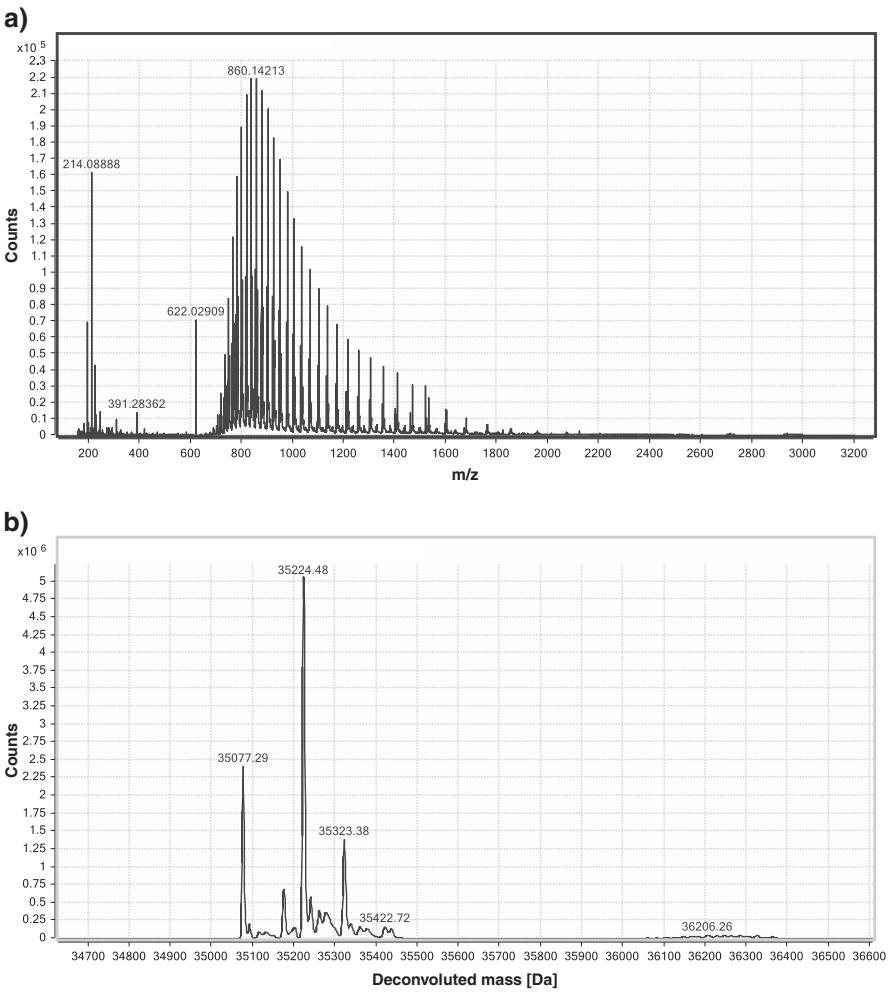

Figure 4. a) ESI-ToF mass spectrum of native WT DFPase; b) Deconvoluted spectrum of native WT DFPase.

conditions, this product distribution is shifted to a higher degree of modification. The dominant species here are those with 3–4 PEG chains. The peak for 5 PEG chains is significantly smaller and higher degrees of modification are again marginal (Figure 5a). Detailed inspection of the first peak in Figure 5a (DFPase with two PEG chains) reveals the polydispersity of the PEG (Figure 5b). The use of 500% conditions leads to reaction mixtures with even slightly higher degrees of modification but species with 3–4 PEG chains remain dominant. Also free PEG was still detected in the mixture forming unspecific complexes with the enzyme. This might explain the sharp decrease in enzymatic activity measured by the pH-stat technique despite the fact that the PEGylation pattern differs only slightly. mPEG-NHS 2000 Da at 50% conditions react with DFPase producing a mixture of conjugates but also un-PEGylated enzyme was detected (Figure 6). The dominance of some specific conjugates is less pronounced compared to mPEG-NHS 750 Da. Species with 2–4 PEG chains attached form the majority of the reaction products but also species with 1 or 5 PEG chains are observed in significant amounts. Even though differences in the PEGylation patterns can be observed between the different PEG reagents these differences are rather small. This is in agreement with other studies that found that the molecular weight of the PEG does only have little effect on the degree of modification.[33,34] ESI-ToF-MS spectra of mPEG-NHS 200 Da used with DFPase at 200% and 500% conditions reveal significant amounts of free PEG still in the mixture and unspecific PEG-protein complexes are formed. This is in agreement with the decreasing enzymatic activity observed under these conditions.

In vivo efficacy of PEGylated DFPase

The nerve agent soman (*O*-pinacolyl methylphosphonofluoridate) was chosen because of the rapid aging of soman-inhibited AChE that renders treatment with reactivating oximes very difficult. As PEGylated DFPase cannot cross the blood-brain barrier rapid detoxification of soman is required before relevant amounts of the agent can reach the rat brain and cause AChE inhibition. The second reason for choosing soman was that the molecule contains two stereocentres leading to four different soman species with drastically different toxicities (Figure 7). The configuration on the phosphorus atom determines whether the soman species is either highly toxic or virtually non-toxic.[35,36]

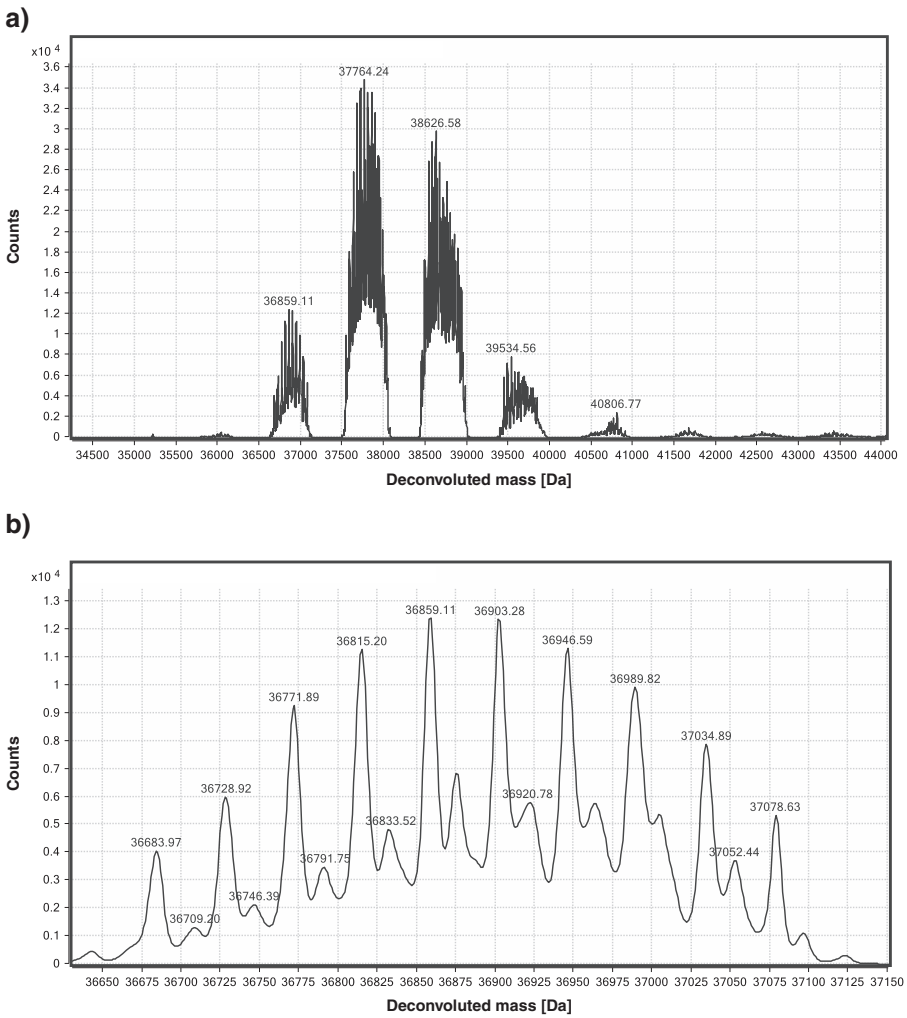

Figure 5. a) Deconvoluted mass spectrum of WT DFPase conjugates with mPEG-NHS (750 Da) at 200% conditions. No un-PEGylated DFPase can be detected and species with 3–4 attached PEG chains dominate the mixture. b) Detailed part of the spectrum shown in a) for the peak centered at 36859.11 Da representing WT DFPase with two attached PEG chains. The polydispersity of the PEG reagent is clearly visible with individual sub-peaks separated one -O-CH_2-CH_2- unit each. Complexity of the peaks arises from the presence of two polydisperse PEG chains.

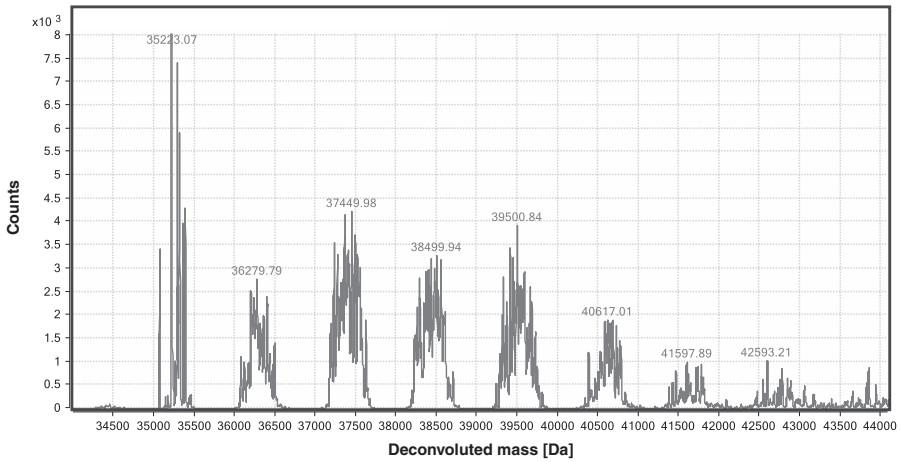

Figure 6. Deconvoluted mass spectrum of conjugates of WT DFPase with mPEG-NHS (2000 Da) at 50% conditions. Even though unreacted DFPase can still be detected the number of attached PEG chains is larger than with mPEG-NHS 750 Da.

To test the *in vivo* efficacy of DFPase to protect rats from the toxic effects of a 3x LD_{50} s.c. injection of soman eight rats were used in a first test with WT DFPase PEGylated with mPEG-NHS 2000 Da (used in 50% concentration as described above but with the filtration steps repeated 20 times). Rats were allocated to four different groups. The first group of three animals was used as a control group and not treated with enzyme but received just the buffer solution (10 mM HEPES, 1 mM $CaCl_2$, 150 mM NaCl, pH 7.4). Two animals were treated with 12.2 mg/kg DFPase ('Normal'). This is the amount of enzyme that is able to degrade the amount of soman injected in a volume equal to the blood volume of the rat with a reaction half-life of 25 s *in vitro*. To calculate the amount of enzyme required the blood volume of the rat was related to its weight using the linear relationship derived by Lee and Blaufox:[37]

$$V_{Blood}[ml] = 0.06\ Weight[g] + 0.77 \quad (1)$$

One animal was treated with 35.8 mg/kg DFPase ('High') and two animals received ca. 71.0 mg/kg DFPase ('Very high'). Five minutes after enzyme prophylaxis soman was injected *s.c.* The control group showed first symptoms of OP poisoning after ca. 3–4 min (intensified grooming and chewing). All animals died within 15 min after injection (12, 14, and 15 min). Animals pretreated with the 'normal' enzyme concentration showed almost identical behaviour. The rat that received the 'high' enzyme concentration also showed first symptoms after 4 min although the time of death was extended to 31 min after soman exposure. Only the two animals receiving the 'very high' enzyme dosage survived. They both showed the early signs of poisoning after 6 and 7 min. WB ChE inhibition 90 min after soman challenge was ca. 69% and 79%. They later showed strong symptoms like heavy salivation and continuous seizures. One of the two surviving animals had to be euthanized after 24 h due to its bad general health conditions (body weight loss of 18.5%). The body weight loss of the other was 13% at day 1 and 20% at day 2 without improvement that led to the supportive infusion of 5% glucose on day 5 and 6 after soman exposure to improve overall health. The animal regained its original body weight only after 13 days. Results are summarized in Table 2.

The outcome of this first test was disappointing as only very high enzyme doses were able to prevent death of the test animal and even at this high DFPase concentration, strong symptoms of OP poisoning were apparent including seizures and most probably seizure-related brain damage as judged by the loss in body weight. One explanation for these results is that the calculation of the enzyme amount required by simply porting *in vitro* data to an *in vivo* case by using the blood volume as the sole compartment in the animal suffers from over-simplification. The pharmacokinetic behaviour of soman after *s.c.* injection is more complicated.[38,39] Another aspect is the preference of the DFPase WT for the less toxic stereoisomers of soman. As previously reported, the rate of soman

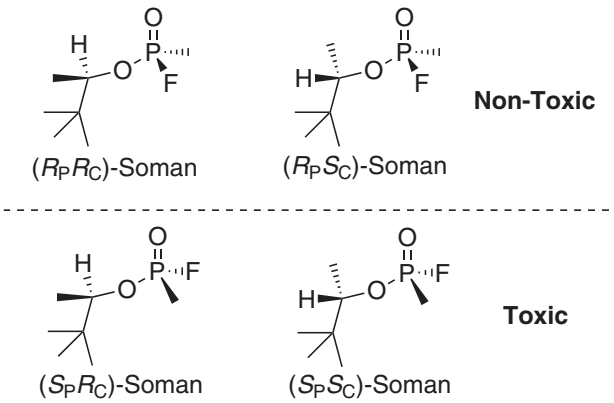

Figure 7. Structures of the four stereoisomers of the nerve agent soman. The two isomers with (S_P)-orientation at the phosphorus atom are toxic while the two isomers with (R_P)-orientation are virtually non-toxic. In aqueous solution racemization can occur over time in a fluoride catalyzed process.

Table 2. Data for animal experiments. Rats were challenged with 3x LD_{50} (*s.c.*) of soman five minutes after the intravenous injection of the PEGylated enzyme (either WT or mutant). *One animal had to be euthanized.

Enzyme dose	Enzyme type	Number of rats	Time to first symptoms	Time to death	survival 24 h	survival > 24 h
[mg/kg]		[N]	[min]	[min]	[%]	[%]
Control	-	3	3, 4, 3	12, 14, 15	0	
12.2	WT	2	3, 3	13, 19	0	
35.8	WT	1	4	31	0	
71.0	WT	2	6, 7	> 24 h	100	50*
Control	-	1	4	10	0	
35.8	Mutant	2	-	-	100	100

degradation does not correspond to the reduction in toxicity and only after most of the virtually non-toxic soman stereoisomers are hydrolyzed a significant hydrolysis of the toxic stereoisomers is observed.[24] The DFPase mutant E37A/R144A/Y146A/T195M displays a reversed enantioselectivity and an enhanced activity against soman. Therefore this mutant was PEGylated with mPEG-NHS 2000 Da and tested *in vivo* to compare its efficacy with that of the DFPase WT. The PEGylation pattern of the mutant was identical to the WT. In this second test only three animals were used. One rat served as a control and only received buffer solution prior to soman injection. The first symptoms were observable after 4 min and death occurred after 10 min. The determination of post-mortem WB ChE activity showed ca. 80% inhibition. The other two rats received a 'high' enzyme dose (35.8 mg/kg) 5 min prior to soman exposure. At this concentration DFPase WT was unable to protect rats from symptoms of OP poisoning and only prolonged the time until death by approx. 15 min. Both rats treated with the DFPase mutant E37A/R144A/Y146A/T195M survived in the experiment. One rat showed slightly increased chewing as the only sign of light OP poisoning while the other rat was virtually free of any observable symptoms. Interestingly, this matched the WB ChE inhibition determined ca. 3 h after challenge, 35 and 24%, respectively. The body weight loss at 24 h was extremely limited in both cases, ca. 3%. Initial body weight was regained between day 9 and 13 for the former, 4 and 6 for the latter. Results are also summarized in Table 2.

The use of mutant E37A/R144A/Y146A/T195M with a preference for the two toxic stereoisomers of soman led to a rapid decrease in toxicity. Even though the number of animals used in both tests is insufficient for statistical analysis the results serve as a proof of principle that DFPase mutants that show preference for the more toxic stereoisomers of soman can protect rats from death and significantly reduce if not avoid symptoms of OP poisoning. Limitations of the currently available mutant are also obvious. Even though enzyme activity (in terms of k_{cat}) against soman is quite high the mutant suffers from an unfavourable K_M value well in the mM range.[24] Given the relatively small soman concentrations found *in vivo* even after a challenge with 3x LD_{50}, the overall efficacy of the enzyme expressed in terms of k_{cat}/K_M is too low. The dose of enzyme mutant administered to the rats would translate into an enzyme dose of 2.9 g for a 70 kg human. Therefore a variant with an improved K_M (while maintaining enantioselectivity and turnover rate) is required to be relevant for practical use. It was reported that a k_{cat}/K_M of 10^7 M^{-1} min^{-1} would be required for detoxifying enzymes.[40] Further enzyme engineering work is directed to achieve this goal.

Immunogenicity of PEGylated DFPase

One major reason to PEGylate therapeutic proteins is to reduce immunogenicity. PEG exhibits a large degree of flexibility in aqueous solution. In addition, it is thought that PEG has the ability to influence the structure of several layers of associated hydrating water molecules. These findings taken together might explain why PEG is remarkably effective in excluding other biopolymers like antibodies, making PEG-protein conjugates both non-immunogenic and non-antigenic.[41]

To test if the administration of PEGylated DFPase leads to the formation of antibodies blood plasma samples were taken after 14 and 27 days from the rats treated with PEGylated mutant E37A/R144A/Y146A/T195M that survived the exposure to soman. An ELISA test was used to determine antibody content. DFPase mutant E37A/R144A/Y146A/T195M was used as the antigen and coated to 96 well-plates. Samples of the rat serum were then applied. To detect antibodies bound to the mutant first a goat anti-rat IgG antibody was applied followed by biotinylated polyclonal rabbit anti-goat immunoglobulins. Finally fluorescein isothiocyanate modified streptavidin was bound to the biotin moiety. The fluorescein label was used for quantification using a Li-Cor Odyssey imaging system. As can be seen from the results (Figure 8) the formation of anti-DFPase antibodies was detected and results were especially strong in the rat that received a second infusion of the enzyme at day 17. Further treatment of this rat with the enzyme would probably not be possible.

Three possible explanations for this outcome can be given. First, the high dose of enzyme given to the rats should be noted. This dose is likely to trigger a stronger response than a significantly lower one. Second, the optimal PEGylation of DFPase might not be achieved using linear NHS-PEG 2000 Da. Using

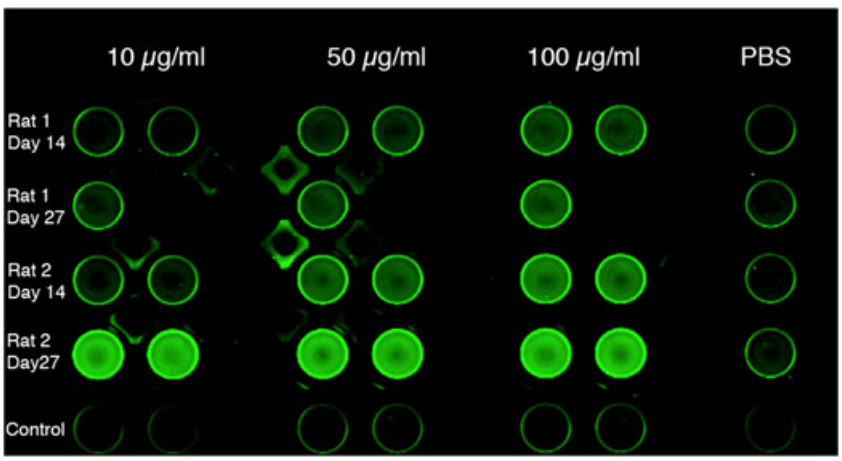

Figure 8. Results of the ELISA aimed to detect antibodies directed against DFPase in rats after treatment with the enzyme. Two rats treated with PEGylated DFPase mutant that survived the soman challenge (Table 2) were sampled 14 and 27 days after exposure. Rat 2 received a second enzyme infusion on day 17 after exposure. Concentrations listed over the columns indicate the concentrations of antigene (DFPase mutant) used for coating the wells of the Maxisorp Immuno 96-well plate. Two controls were used. One in which only PBS buffer was used for coating (last column) and the other in which PBS buffer was used instead of rat plasma (last row). Stronger fluorescence intensity indicates higher concentrations of anti-DFPase antibodies.

higher molecular weight PEGs and/or branched PEG structures might result in a more effective shielding of the molecular surface of DFPase and therefore lower immune response. This optimization is the focus of current work. Third, a mixture of different PEGylated conjugates was employed. Some un-PEGylated DFPase might still be present in the administered dose as PEGylated species were not separated. Future tests will therefore depend on the separation of individual conjugates.

Conclusion

The results of the present study clearly demonstrate that DFPase can be grafted with several PEG chains without a sharp decrease in enzymatic activity. However only up to eight PEG chains per DFPase molecule were observed using ESI-ToF-MS techniques. This is attributed to the fact that PEG chains form a shell-like structure around the protein surface therefore hindering the access of further mPEG-NHS molecules to the reactive lysine residues of the protein. *In vivo* experiments of PEGylated WT DFPase in rats challenged with a 3x LD_{50} (*s.c.*) dose of soman showed that only extremely high amounts of enzyme can maintain life and even at that dose strong symptoms of OP poisoning were apparent. This is attributed to the selectivity of WT DFPase for the almost non-toxic stereoisomers of soman, which leads to hydrolysis of the agent without a reduction in toxicity. Experiments with PEGylated DFPase mutant E37A/Y144A/R146A/T195M however showed more promising results, as it was possible to keep rats alive without or with only very mild initial symptoms of poisoning detectable. Despite the fact that high doses of enzyme were required and rats developed anti-DFPase antibodies after a number of days, this is a clear proof of principle that a DFPase mutant engineered for preference of the more toxic stereoisomers of the nerve agents and with an improved substrate affinity can be used as a potential *in vivo* bioscavenger against poisoning with OP nerve agents.

Acknowledgments

This work was partly supported the German Ministry of Defense under contract number E/UR3G/6 G115/6A801. Part of this work was carried out by M. Melzer in partial fulfillment of the requirements for a medical doctoral degree at the Johannes Gutenberg University, Mainz, Germany. Ms Annie Foquin's technical skills are greatly acknowledged for the *in vivo* experiments performed at CRSSA. M.M. Blum acknowledges the US Department of Energy and Los Alamos National Security for a Director's Fellowship at the Los Alamos National Laboratory. (LA-UR 11-04147)

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