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MOLECULAR TOXICOLOGY

Efficacy of the rePON1 mutant IIG1 to prevent cyclosarin toxicity in vivo and to detoxify structurally different nerve agents in vitro

Franz Worek · Thomas Seeger · Moshe Goldsmith · Yacov Ashani · Haim Leader · Joel S. Sussman · Dan Tawfik · Horst Thiermann · Timo Wille

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Abstract The potent human toxicity of organophosphorus (OP) nerve agents calls for the development of effective antidotes. Standard treatment for nerve agent poisoning with atropine and an oxime has a limited efficacy. An alternative approach is the development of catalytic bioscavengers using OP-hydrolyzing enzymes such as paraoxonases (PON1). Recently, a chimeric PON1 mutant, IIG1, was engineered toward the hydrolysis of the toxic isomers of soman and cyclosarin with high in vitro catalytic efficiency. In order to investigate the suitability of IIG1 as a catalytic bioscavenger, an in vivo guinea pig model was established to determine the protective effect of IIG1 against the highly toxic nerve agent cyclosarin. Prophylactic i.v. injection of IIG1 (1 mg/kg) prevented systemic toxicity in cyclosarin (~2LD_{50})-poisoned guinea pigs, preserved brain acetylcholinesterase (AChE) activity, and protected erythrocyte AChE activity partially. A lower IIG1 dose (0.2 mg/kg) already prevented mortality and reduced systemic toxicity. IIG1 exhibited a high catalytic efficiency with a homologous series of alkylmethylfluorophosphonates but had low efficiency with the phosphoramidate tabun and was virtually ineffective with the nerve agent VX. This quantitative analysis validated the model for predicting in vivo protection by catalytic bioscavengers based on their catalytic efficiency, the level of circulating enzyme, and the dose of the intoxicating nerve agent. The in vitro and in vivo results indicate that IIG1 may be considered as a promising candidate bioscavenger to protect against the toxic effects of a range of highly toxic nerve agents.

Keywords Nerve agents · Paraoxonase · Mutant · Detoxification · Protection · Bioscavenger

F. Worek (✉) · T. Seeger · H. Thiermann · T. Wille
Bundeswehr Institute of Pharmacology and Toxicology, Neuherbergstrasse 11, 80937 Munich, Germany
e-mail: franzworek@bundeswehr.org

M. Goldsmith · Y. Ashani · D. Tawfik
Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, Israel

H. Leader
Department of Materials and Interfaces, Weizmann Institute of Science, Rehovot, Israel

J. S. Sussman
Department of Structural Biology, Weizmann Institute of Science, Rehovot, Israel

Introduction

The high human toxicity of a class of organophosphorus (OP) compounds developed for use as chemical warfare nerve agents calls for the development of effective antidotes (Marrs 2007; Gunnell et al. 2007). The acute toxic effects of OP nerve agents are caused by covalent binding to the active site serine of the pivotal enzyme acetylcholinesterase (AChE) resulting in the accumulation of the neurotransmitter acetylcholine at muscarinic and nicotinic synapses (Aldridge and Reiner 1972; Taylor et al. 1995). In the end, this causes an overstimulation of peripheral and central cholinergic receptors and leads ultimately to an impairment of vital body functions and finally to respiratory arrest and death (Grob 1956; Lee 2003; Kwong 2002).

The standard treatment of poisoning by OP nerve agents includes the muscarinic antagonist atropine and an oxime, e.g., obidoxime or pralidoxime (Cannard 2006; Johnson et al. 2000). Hereby, atropine acts as a symptomatic antidote at muscarinic receptors and oximes may provide

causal therapy by reactivating OP-inhibited AChE (Eyer and Worek 2007). However, the standard treatment has limited efficacy in case of poisoning by various nerve agents, e.g., soman, tabun, and cyclosarin and is inadequate to prevent incapacitation (Worek and Thiermann 2013).

This disappointing situation led to research on alternative approaches directed to minimize or prevent systemic effects of nerve agents (Doctor et al. 1991; Lenz et al. 2007). Ongoing research is conducted on enzyme-based scavengers that bind (stoichiometric) or hydrolyze (catalytic) nerve agents before they can bind to synaptic AChE (Masson and Rochu 2009; Mumford et al. 2013). Human butyrylcholinesterase (BChE) has been developed as a stoichiometric scavenger for pre- and post-exposure use (Brandeis et al. 1993; Allon et al. 1998; Mumford and Troyer 2011). However, large BChE doses are required to bind lethal nerve agent concentrations (Allon et al. 1998). In contrast, lower doses would be needed with catalytic scavengers. In fact, several OP-degrading enzymes including mammalian paraoxonase (PON1), *Pseudomonas diminuta* organophosphorus hydrolase (OPH), *Alteromonas* prolidase organophosphorus acid anhydrase (OPAA), and *Loligo vulgaris* diisopropylfluorophosphatase (DFPase) have been identified and characterized (Masson and Rochu 2009; Dumas et al. 1989; Hartleib and Rüterjans 2001).

All nerve agents possess one (e.g., sarin, cyclosarin, VX, and tabun) or two chiral centers (soman), and the enantiomers differ in their toxicity and biological stability (Benschop and de Jong 1988; Reiter et al. 2008; Tenberken et al. 2010). However, OP-hydrolyzing enzymes detoxify preferentially the less toxic P(+) enantiomers and exhibit at most a low catalytic efficacy with the toxic P(−) enantiomers (Masson and Rochu 2009; diTargiani et al. 2010; Otto et al. 2013). In fact, toxicokinetic studies have shown that the less toxic isomers of G-type nerve agents are rapidly eliminated, presumably via enzymatic degradation, while the toxic enantiomers are more stable in the circulation (Spruit et al. 2000).

Recently, a chimeric PON1 mutant, IIG1, with reversed stereoselectivity was obtained using directed evolution and showed high catalytic efficacy against soman and cyclosarin in vitro (Goldsmith et al. 2012). Having k_{cat}/K_M values $>3 \times 10^7\ M^{-1}\ min^{-1}$ when reacting with soman and cyclosarin, IIG1 was considered an appropriate candidate for in vivo testing. To investigate the suitability of IIG1 as a catalytic bioscavenger, an in vivo guinea pig model was established to determine the protective effect of IIG1 against the highly toxic nerve agent cyclosarin. In addition, the detoxification kinetics of IIG1 with a homologous series of alkylmethylphosphonates and other nerve agents (Fig. 1) was determined in vitro in order to assess the ability of this enzyme to detoxify a broad spectrum of structurally diverse nerve agents.

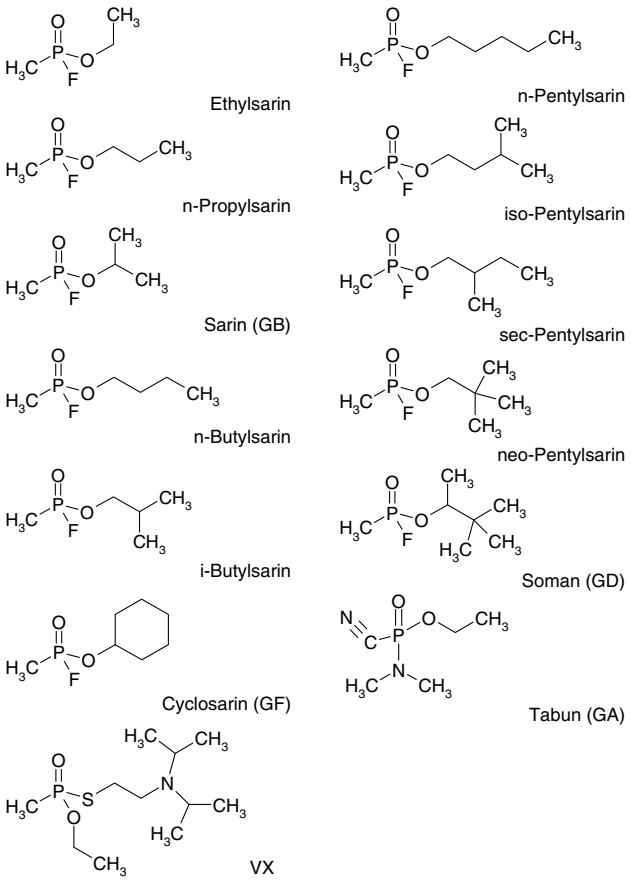

Fig. 1 Chemical structures of the tested nerve agents

Materials and methods

Chemicals

Racemic OP nerve agents (Fig. 1; >98 % by GC–MS, ^{1}H NMR and ^{31}P NMR) were made available by the German Ministry of Defense. Triton X-100, 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).

Nerve agent stock solutions (1 % v/v) were prepared in acetonitrile and were stored at ambient temperature. Cyclosarin working solution (200 μg/ml) for in vivo testing was prepared for each experiment in saline and was kept on ice until use.

Enzyme expression and purification

rePON1 variant, IIG1, was expressed in Origami B DE3 cells and purified using Ni–NTA chromatography at the Israel Structural Proteomics Center (ISPC), Weizmann Institute of Science, Rehovot, Israel, in accordance with the procedures described by Gupta et al. (2011) and Goldsmith

et al. (2012). The purified protein solution was dialyzed extensively, before use, against an isotonic activity buffer (50 mM Tris–HCl pH 8.0, 1 mM $CaCl_2$, 100 mM NaCl, and 0.02 % Tergitol NP-10). Protein purity was >95 % (SDS–PAGE) and its concentration was determined by the colorimetric BCA method (1.37 ± 0.044 mg/ml). To assay for in vivo toxicity and pyrogenicity, the protein was injected (i.v.) to C57BL/6 J mice at doses of up to 5 mg/kg. No signs or symptoms were observed for at least two weeks.

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 laboratory chow and water. Animals were allowed to accustom to the facility for at least 1 week before starting experiments. The experimental protocol was approved by the institutional ethics committee.

Experimental procedure

The guinea pigs were anesthetized by i.m. injection of a mixture of medetomidin (0.2 mg/kg), midazolam (1.0 mg/kg), and fentanyl (0.025 mg/kg). 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 trachea, right v. jugularis, and left a. carotis were prepared, and a tracheal cannula and catheters were inserted and fixed.

The animals were randomly divided into four groups ($n = 6$–7):

- Saline control
- GF control
- IIG1 (0.2 mg/kg) prophylaxis and GF exposure
- IIG1 (1.0 mg/kg) prophylaxis and GF exposure

At −60 min, IIG1 (0.2 or 1.0 mg/kg i.v.) or saline and at 0 min, cyclosarin (GF; 100 µg/kg s.c.) or saline were administered. The injection volume was 0.5 ml/kg throughout. Multiple blood samples (350 µl, the volume was replaced by saline) were taken between −61 and +120 min for the measurement of AChE activities and IIG1 levels. An aliquot was immediately diluted 20-fold in ice-cold distilled water, vortex mixed, shock-frozen in liquid nitrogen, and stored at −80 °C until measurement of AChE activity. IIG1 level in plasma was determined by CMP-coumarin hydrolysis as previously described (Gupta et al. 2011). Briefly, the initial velocity was monitored at 405 nm following 20 µl plasma dilution in 1 ml 50 mM Tris–HCl pH 8.0, 1 mM $CaCl_2$, containing 0.1 % Tergitol, and 60 µM CMP-coumarin. A predetermined calibration curve generated by adding increasing amounts of IIG1 to equal volume of assay sample containing 20 µl of naïve animal plasma was used to calculate IIG1 in plasma samples. Guinea pigs plasma is devoid of CMP-coumarin hydrolytic activity.

At 120 min or death of the animal, the brain was removed; immediately dissected into frontal cortex, striatum, hippocampus, and medulla; shock-frozen in liquid nitrogen; and stored at −80 °C until measurement of AChE activity.

Clinical signs and symptoms were visually recorded and scored into local fasciculations at injection site, local or generalized convulsions, respiratory depression (abnormal rhythm or frequency), respiratory arrest, and death.

AChE assay

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

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

Data analysis

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

In vitro degradation of OP by IIG1

The degradation kinetics of nerve agents (Fig. 1) by IIG1 were investigated with an AChE inhibition assay, using

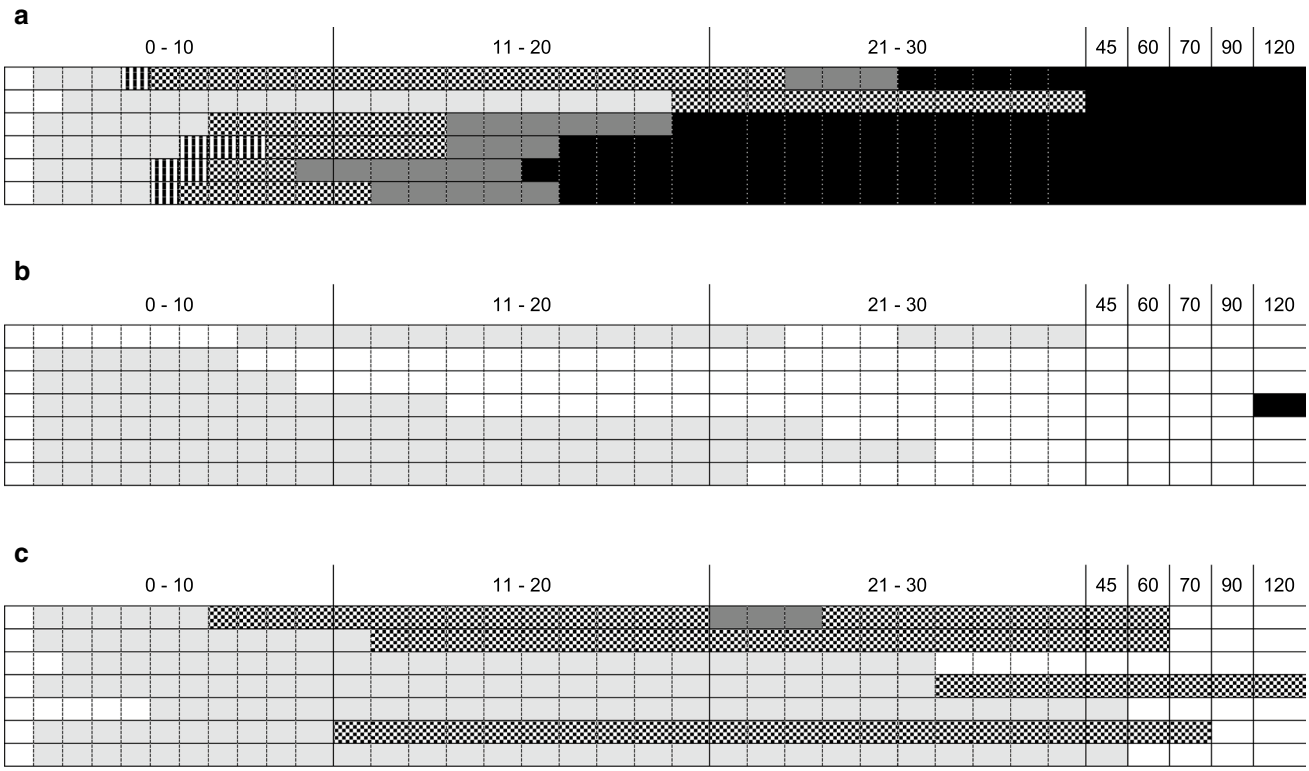

Fig. 2 Clinical signs and symptoms and survival of GF-poisoned guinea pigs. *Each line* represents a single animal and the time is given in minutes. No symptoms (*white*), local fasciculations (*light grey*), local or generalized convulsions (*vertical lines*), respiratory depression (*cross hatched*), respiratory arrest (*dark grey*) and death (*black*). **a** 100 µg/kg GF s.c., **b** 1.0 mg/kg IIG1 i.v. 60 min prior to 100 µg/kg GF s.c., **c** 0.2 mg/kg IIG1 i.v. 60 min prior to 100 µg/kg GF s.c.

human erythrocyte ghosts as AChE source (Worek et al. 2004). Nerve agents and IIG1 were appropriately diluted in isotonic buffer (50 mM Tris–HCl pH 8.0, 1 mM $CaCl_2$, 100 mM NaCl and 0.02 % Tergitol Np-10). About 10 µl IIG1 was mixed with 50 µl nerve agent dilution resulting in the final concentrations of 20 µM (*i*-butylsarin, pentylsarin analogues), 25 µM (VX, cyclosarin, *n*-propylsarin, *n*-butylsarin), 50 µM (soman), 80 µM (sarin), 125 µM (ethylsarin), and 500 µM (tabun) and were incubated at 37 °C, pH 7.40. At defined time points, 5 µl samples were taken and added to a pre-warmed (37 °C) polystyrol cuvette that previously had been filled with 3,000 µl phosphate buffer (0.1 M; pH 7.4), 100 µl DTNB (0.3 mM final concentration), and 50 µl ATCh (0.45 mM final concentration). Immediately, thereafter, 10 µl AChE was added and the AChE activity was measured.

The recorded AChE inhibition curves were analyzed by nonlinear regression analysis to determine the first-order inhibition rate constant k_1 (Aurbek et al. 2006). Then, k_1 was plotted against time of incubation of the OP with IIG1, to calculate the first-order degradation constant k_{obs}. Based on the classical Michaelis–Menten equation and assuming $K_M/[OP] > 10$, the second-order degradation constant k_{cat}/K_M was calculated according to Eq. 1.

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

The analysis of the data and the calculation of the kinetic constants by nonlinear regression analysis were performed with GraphPad Prism 4.03. The data of duplicate experiments are given as mean ± SD.

Results

GF poisoning in vivo

The subcutaneous injection of 100 µg/kg GF (~2LD_{50}) resulted in a rapid onset of signs and symptoms of cholinergic overstimulation (Fig. 2a). Local fasciculations became visible during the slow (1 min) injection of GF. Clonic convulsions of the lower limbs developed within a few minutes, followed by generalized convulsions. Then, respiratory depression, i.e., reduced frequency, deep breathing, and gasping, became obvious and was followed by respiratory arrest in almost all animals. This was followed by cardiac arrest and death a few minutes later. The animals died between 16 and 37 min after GF injection (22 ± 7.5 min).

IIG1 prophylaxis in vivo

The i.v. injection of IIG1 (0.2 or 1.0 mg/kg) did not results in overt signs and symptoms and had no effect on erythrocyte AChE activity.

The i.v. injection of 1.0 mg/kg IIG1 60 min prior to s.c. GF (100 μg/kg) completely prevented systemic signs of poisoning (Fig. 2b). Only fasciculations at the injections site were recorded. Six out of seven animals survived until the end of the observation period (120 min). One animal died immediately after the 90-min blood sampling due to iatrogenic pulmonary embolism.

Prophylactic administration of a reduced IIG1 dose (0.2 mg/kg i.v.) prevented lethality in all animals (Fig. 2c). Four animals suffered from intermediate respiratory depression (reduced frequency, deep breathing, and gasping) while in three animals, only fasciculations at the injection sites were visible.

IIG1 time course in guinea pigs

Analysis of IIG1 plasma concentration over time (180 min) indicated that the enzyme redistributed rapidly following i.v. load and produced a stable circulating level of IIG1 activity throughout the entire experiment (Fig. 3). Thus, in the first 60-min post-enzyme load, a mean (±SE; $n = 7$) of 12.13 ± 0.24 and 3.17 ± 0.11 μg/ml IIG1 in plasma was established following 1.0 and 0.2 mg/kg, respectively. These values correspond to 0.30 and 0.08 μM IIG1 prior to exposure to GF. The injection of GF did not seem to significantly affect IIG1 plasma levels. The rapid equilibration of IIG1 activity at approximately 50 % of the administered dose (assuming 39 ml plasma/kg; Ancill 1956) together with the circulatory stability is similar to a previously described rePON1 (Gupta et al. 2011), possibly owing to the association with the HDL fraction is involved (Gaidukov and Tawfik 2005). Thus, it is suggested that IIG1 may provide protection against lethal doses of GF well above the time course covered in this study.

Erythrocyte AChE activity

The pre-exposure erythrocyte AChE activities were 178 ± 19 mU/μmol Hb (control group), 163 ± 19 mU/μmol Hb (GF group), 170 ± 22 mU/μmol Hb (1.0 mg/kg IIG1 group), and 160 ± 14 mU/μmol Hb (0.2 mg/kg IIG1 group).

Poisoning by GF resulted in a rapid and complete inhibition of erythrocyte AChE activity (Fig. 4a). Prophylactic administration of 1.0 mg/kg IIG1 reduced the decrease in enzyme activity and preserved approximately 40 % of active AChE. The AChE values of the IIG1 group were significantly higher compared to those of the GF group

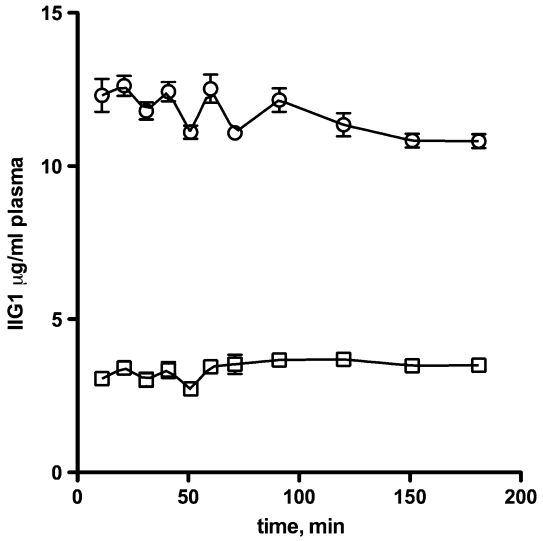

Fig. 3 Plasma concentration of IIG1 over time. Data points are averages (±SE) from seven animals (duplicate assays per each plasma sample). About 1 mg/kg (*circles*) or 0.2 mg/kg (*squares*) IIG1 was administered i.v. at $t = 0$, and animal was challenged with approximately 2 × LD_{50} GF at $t = 60$ min

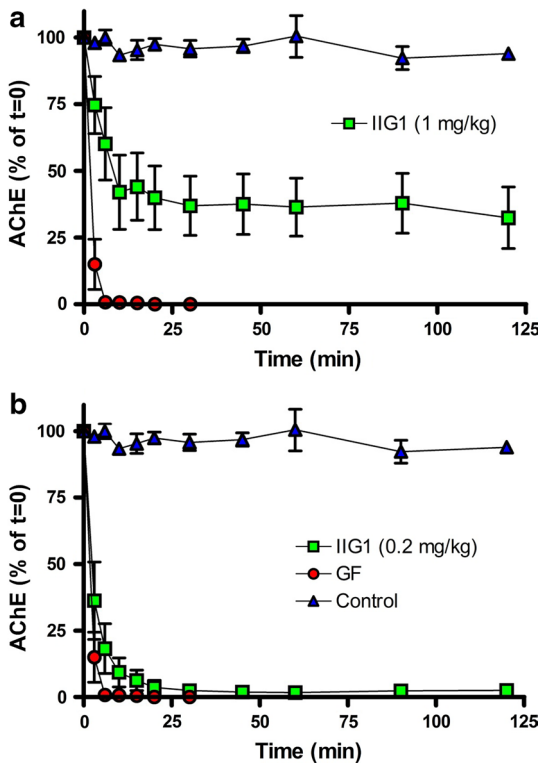

Fig. 4 Time- and treatment-dependent changes of erythrocyte AChE activity of control, IIG1-, and GF-treated guinea pigs. Data are given as % of pre-exposure control AChE activity as mean ± SD. Identical symbols for control and GF groups were used in **a** and **b**

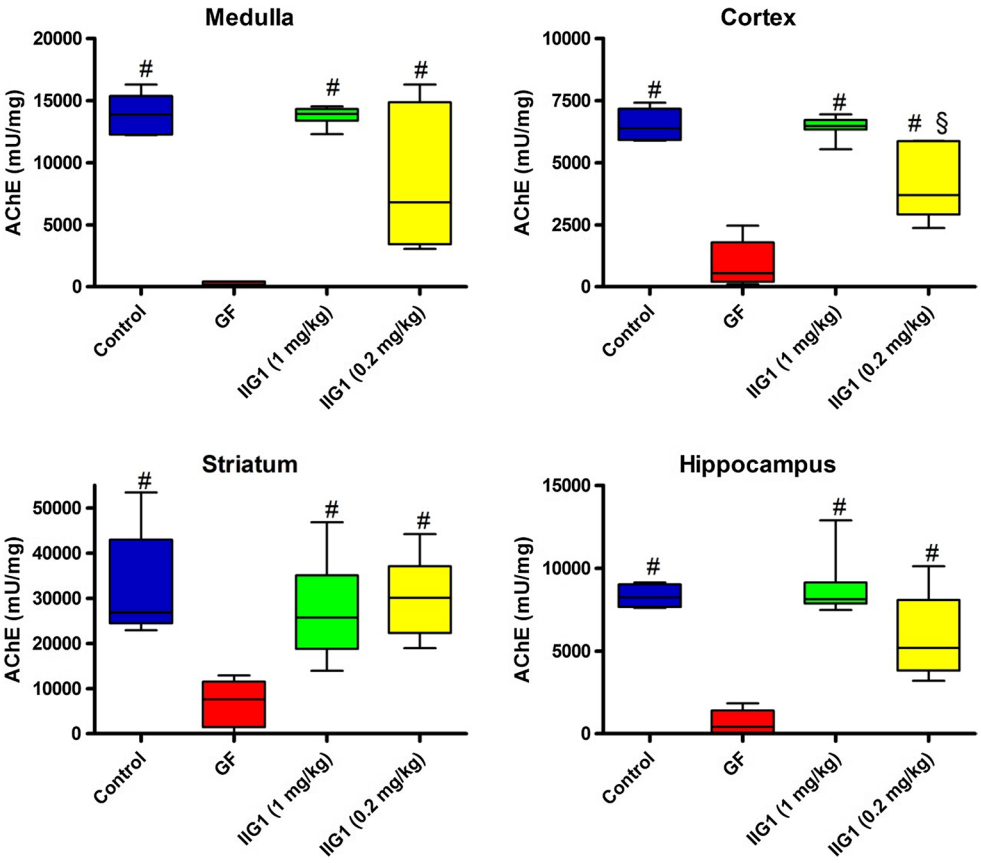

Fig. 5 Brain AChE activity of control, IIG1-, and GF-treated guinea pigs. Data of medulla, cortex, striatum, and hippocampus AChE activity (mU/mg) are given as mean ± SD. $^{\#}p < 0.05$ to GF group; $^{\S}p < 0.05$ to control group

throughout the experiment and were significantly lower compared to control from 6 to 120 min.

Administration of 0.2 mg/kg IIG1 slowed down the decrease in erythrocyte AChE slightly but did not prevent almost complete inhibition of the enzyme (Fig. 4b).

Brain AChE activity

GF poisoning induced an almost complete inhibition of brain AChE activity, which was determined separately in medulla oblongata, frontal cortex, striatum, and hippocampus (Fig. 5). Prophylaxis with 1.0 mg/kg IIG1 prevented brain AChE inhibition, and the values were not different from control AChE activity. Pre-treatment with the lower IIG1 dose (0.2 mg/kg) preserved AChE activity in medulla, striatum, and hippocampus but was significantly lower compared to control in cortex.

In vitro detoxification of OP by IIG1

The detoxification of a homologous series of alkylmethylfluorophosphonates, VX, and tabun (Fig. 1) by IIG1 was investigated with a biological assay. Incubation of IIG1 with appropriate nerve agent concentrations and transfer of aliquots into cuvets for the online recording of AChE inhibition after various time allowed the calculation of the detoxification kinetics quantified as k_{obs} (Fig. 6). The catalytic efficacy of IIG1 was quantified by the calculated k_{cat}/K_M (Eq. 1). IIG1 was most active with cyclosarin (GF) followed by pentylsarin analogues and soman (Table 1). The PON1 mutant showed a substantially lower efficacy with nerve agents bearing shorter alkyl residues, i.e., ethyl, propyl, and butyl. IIG1 had a very low catalytic efficacy with the phosphoramidate tabun and a negligible effect with VX (Table 1; VX experiments performed under second-order conditions).

Discussion

IIG1 in vivo efficacy

Previously, as low as 15 μg/mouse wt OPH from *P. diminuta* was reported to confer alone protective ratio of four against tabun intoxication without supporting drugs (Raveh et al. 1992). The results of the present study demonstrated for the first time the prophylactic efficacy of the chimeric PON1 mutant IIG1 against the highly toxic nerve agent cyclosarin (GF) in vivo. Cyclosarin is a highly potent inhibitor of AChE from different species with its

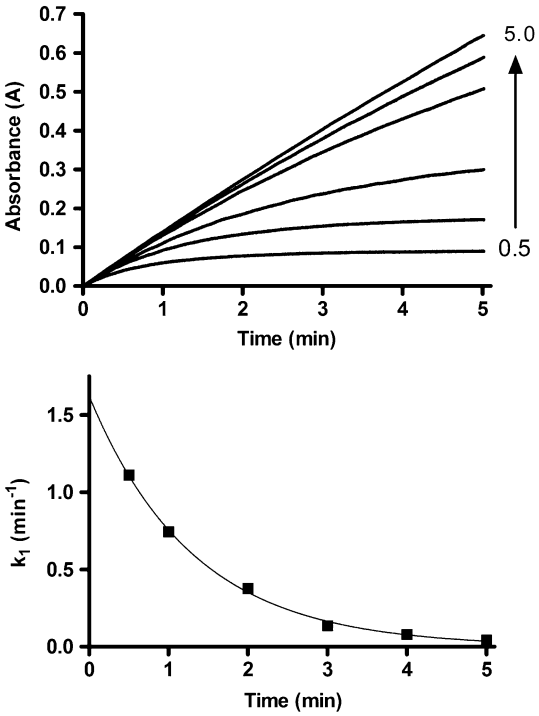

Fig. 6 Time-dependent detoxification of soman (GD) by IIG1. Original registration of the time-dependent (0.5–5 min) decrease in AChE inhibition by GD after incubation with IIG1. Secondary plot of k_1 versus time for the determination of the detoxification velocity (k_{obs})

Table 1 Detoxification of nerve agents by IIG1

Code	k_{cat}/K_M (M^{-1} min^{-1}) mean ± SD	IIG1 (nM)	Ratio to GF (%)
Ethylsarin	$9.5 \pm 0.13 \times 10^6$	11.2	11
n-Propylsarin	$1.6 \pm 0.12 \times 10^7$	11.2	19
Sarin (GB)	$9.5 \pm 1.25 \times 10^6$	11.2	11
n-Butylsarin	$2.3 \pm 0.11 \times 10^7$	11.2	28
iso-Butylsarin	$3.0 \pm 0.05 \times 10^7$	11.2	36
n-Pentylsarin	$7.7 \pm 0.16 \times 10^7$	5.6	92
iso-Pentylsarin	$5.1 \pm 0.66 \times 10^7$	5.6	61
sec-Pentylsarin	$5.2 \pm 0.48 \times 10^7$	5.6	62
neo-Pentylsarin	$3.6 \pm 0.32 \times 10^7$	5.6	43
Cyclosarin (GF)	$8.4 \pm 0.85 \times 10^7$	5.6	100
Soman (GD)	$6.4 \pm 0.85 \times 10^7$	11.2	76
Tabun (GA)	$2.6 \pm 0.30 \times 10^6$	11.2	3
VX	$1.43 \pm 0.05 \times 10^2$	33,600	0

k_{cat}/K_M (M^{-1} min^{-1}) values are given as mean ± SD of two independent experiments. IIG1 concentration in nM used during incubation with nerve agents

second-order inhibition rate constant k_i being substantially higher compared to k_i of other relevant G-type nerve agents, tabun, sarin, and soman (Worek and Thiermann 2013). In addition, cyclosarin-inhibited AChE was shown to be rather resistant toward reactivation by clinically used oximes, e.g., obidoxime and pralidoxime (Worek et al. 2004; Worek et al. 2002), which translates into inadequate reactivation of cyclosarin-inhibited AChE in vivo and in a limited therapeutic efficacy of these oximes in different species (Clement 1992; Shih et al. 2009; Luo and Liang 1997; Lundy et al. 1992).

Wild-type mammalian (human) PON1 has a limited catalytic efficacy against G-type nerve agents, i.e., soman, sarin, cyclosarin, and tabun and provides inadequate protection from nerve agent toxicity in poisoned animals (Amitai et al. 2006; Kirby et al. 2013; Josse et al. 2001; Hodgins et al. 2013). This is partly due to its unfavorable stereoselectivity, i.e., the preferential degradation of the less toxic P(+) nerve agent stereoisomer (Amitai et al. 2006). In contrast, the evolved rePON1 mutant, IIG1, showed high catalytic activity against the toxic, S_P stereoisomers of sarin, soman, and cyclosarin (Goldsmith et al. 2012). Having a k_{cat}/K_M of 3.4×10^7 M^{-1} min^{-1} against S_P-cyclosarin, this enzyme fulfilled the previously defined minimal requirement for a catalytic bioscavenger, i.e., $k_{cat}/K_M > 1 \times 10^7$ M^{-1} min^{-1} (Gupta et al. 2011).

In fact, the prophylactic administration of 1.0 mg/kg IIG1 prevented systemic toxic signs and symptoms in guinea pigs poisoned by 100 μg/kg cyclosarin (Fig. 2b). This cyclosarin dose should resemble $2LD_{50}$ according to previously determined LD_{50} values of 40–57 μg/kg (Lundy et al. 1992; Shih et al. 2011; Wetherell et al. 2006). However, these values were determined in conscious guinea pigs, and the rapid onset of toxic signs together with the short survival time of 16–37 min indicates that the selected dose was in fact supralethal in our experimental setup with anesthetized guinea pigs.

The beneficial effect of IIG1 was obviously due to rapid catalytic detoxification of cyclosarin, as demonstrated by the in vitro kinetic measurements. Thus, the administered cyclosarin dose of 100 μg/kg, which is ~560 nmol/kg, was hydrolyzed rapidly by 1.0 mg/kg IIG1 that corresponds to ~25 nmol/kg only. More specifically, calculations suggest that at the time of GF injection, the 1 mg/kg dose produced in plasma 0.3 μM IIG1 (Fig. 3) that is theoretically expected to hydrolyze >96 % of the toxic isomer of GF in approximately 8 s (assuming rapid and homogenous distribution of GF in plasma; $[t_{1/2} = 0.69/(8.4 \times 10^7 \times 0.3 \times 10^{-6}) = 1.64$ s]). In the case of the 0.2 mg/kg, 75 % of the available GF will be hydrolyzed within 12 s. Thus, it seems that catalytic scavenger that can decrease the OP levels in blood to below 1/10 of its LD_{50} dose within 10 s is likely to provide sign-free protected animals. In fact, since the rate of complete entry of GF into the circulation following s.c. injection requires several minutes, the above estimates are an upper-limit values for the time

required to detoxify 2 × LD_{50} GF before it reaches the target organs. Indeed, the rapid detoxification of cyclosarin in the systemic circulation that contains the 1 mg/kg dose of IIG1 minimized distribution of the agent into target tissues and thus prevented toxic effects. Hence, no visible signs of poisoning, i.e., convulsions or respiratory depression (Fig. 2b), could be observed, and brain AChE activity was not different from control (Fig. 5). This assumption is further supported by the fact that erythrocyte AChE activity was only partially inhibited. In theory, if the GF dose used could rapidly and homogeneously mix in vivo with the whole guinea pig blood volume (79 ml/kg; Ancill 1956), the presence of 0.3 μM IIG1 (Fig. 3) produced from the 1 mg/kg dose would not be able to provide any protection to RBC–AChE. This theoretical estimate (Ashani et al. 1972) is based on the bimolecular rate constant of the inhibition of guinea pig AChE by GF ($k_i = 1.8 \times 10^8$ M^{-1} min^{-1}; Worek and Thiermann 2013), k_{cat}/K_M of GF hydrolysis by IIG1 (8.4×10^7 M^{-1} min^{-1}) and the concentrations of GF (assumed 7 μM) and IIG1 (0.3 μM). The finding that 40 % of guinea pig blood AChE were protected is attributed to the relatively slow rate of diffusion compared to an instantaneous mix and presumably incomplete absorption of GF from the s.c. injection site. The fact that the toxic isomer of inhaled sarin peaked in guinea pig blood several minutes after the initiation of the inhalation of 0.8 × LCt_{50} dose (Spruit et al. 2000) suggests that the s.c. exposure model is adequate to provide proof of concept for the protection conferred by catalytic scavengers. Obviously, it will be interesting to evaluate IIG1 efficacy in both i.v. injected and nose-only-exposed animals to vapors of GF.

Injection of a fivefold lower IIG1 dose prevented lethality but could not circumvent systemic toxicity (Fig. 2c). Based on the above-mentioned considerations, the IIG1 concentration was obviously too low to detoxify cyclosarin in the systemic circulation fast enough to prevent transfer of cyclosarin into tissue. Nevertheless, systemic toxicity was reduced and six out of seven animals recovered totally until end of the observation period.

The possibility to apply the biochemical concept of the detoxification in vitro (k_{cat}/K_M) to predict protection in vivo is highly advantageous. Thus, knowing k_{cat}/K_M and the level of the circulating enzyme seems to be sufficient to estimate the rate of detoxification in blood within one blood circulating time and estimate the decrease in toxic concentrations of nerve agents, over short period of time, so as to prevent its transfer to target tissues. The results presented here with IIG1 in guinea pigs indeed suggest that the catalytic proficiency and enzyme blood concentrations can be considered as reliable parameters for such a quantitative prediction and thus decrease significantly the number of animal experiments that are required to establish dose–efficacy relationship. Further, the extrapolation from animals to human should be a most trustworthy protocol.

Catalytic efficacy of IIG1 in vitro

The initial characterization of the degradation kinetics of in situ generated nerve agents by IIG1 indicated that this enzyme had the highest catalytic efficacy with soman, closely followed by cyclosarin while the k_{cat}/K_M was more than tenfold lower with sarin (Goldsmith et al. 2012). In order to investigate further the effects of nerve agent structure on IIG1′s hydrolytic activity, we determined the detoxification kinetics of IIG1 with a series of alkylmethylfluorophosphonates, the phosphoramidate tabun, and the phosphonothionate VX (Fig. 1). By using an AChE inhibition assay, the ability of IIG1 to detoxify the toxic stereoisomers could be quantified. It became evident that the catalytic efficacy of IIG1 was dependent on the structure of the alkyl side chain of the alkylmethylfluorophosphonates (Table 1).

IIG1 detoxified preferentially compounds with bulky side chains showing the highest k_{cat}/K_M values with cyclosarin while its effect was substantially lower with sarin and its *n*-propyl and ethyl analogues. Nevertheless, the k_{cat}/K_M was > 1×10^7 M^{-1} min^{-1} with most of the agents. The catalytic efficacy of IIG1 was markedly lower with tabun (k_{cat}/K_M of 2.6×10^6 M^{-1} min^{-1}). However, due to the 25-fold lower inhibitory potency of tabun toward human AChE (Worek and Thiermann 2013) and its lower in vivo toxicity, a protective effect may be anticipated at sufficiently high IIG1 doses.

Previous studies showed that wild-type PON1 does not degrade the phosphonothionate VX (Masson et al. 1998) and in fact IIG1 hardly detoxified VX even at conditions with a molar excess of IIG1 (Table 1). Notably, the k_{cat}/K_M obtained with authentic cyclosarin, soman, and sarin at 37 °C is consistent with the values reported by Goldsmith et al. (2012) at 25 °C, who used in situ generated G-type nerve agents.

Conclusions

The first investigation of the prophylactic effect of the chimeric PON1 mutant IIG1 in vivo demonstrated a protective effect of this enzyme against the highly toxic nerve agent cyclosarin in guinea pigs. The high catalytic efficacy of IIG1 together with its blood levels obviously detoxified cyclosarin rapidly enough to minimize the distribution of this agent into target tissues. A reasonable correlation was demonstrated between the administered dose of IIG1 and level of protection conferred following GF challenge. The quantitative analysis suggests a desirable goal of k_{cat}/K_M of

approximately 5×10^7 M^{-1} min^{-1} to achieve protection with less than 1 mg/kg of a catalytic scavenger. In addition, IIG1 showed a high catalytic efficacy with a series of alkylmethylphosphonofluoridates, i.e., agents that could be used as nerve agents. The kinetics were less favorable with tabun; yet, IIG1 may have a beneficial effect against this agent in vivo if administered at a sufficiently high dose. Comparable to wild-type and other PON1 mutants, IIG1 showed a negligible effect against VX. In the end, IIG1 may be considered as an effective detoxifying enzyme with a broad spectrum against fluorophosphonates and a phosphoramidate. Thus, the in vitro and in vivo results indicate that IIG1 may be considered as a promising candidate bioscavenger to protect against the toxic effects of a range of highly toxic nerve agents.

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Conflict of interest The authors declare that there are no conflicts of interest.

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