Progress in the development of enzyme-based nerve agent bioscavengers

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Acetylcholinesterase is the physiological target for acute toxicity of nerve agents. Attempts to protect acetylcholinesterase from phosphylation by nerve agents, is currently achieved by reversible inhibitors that transiently mask the enzyme active site. This approach either protects only peripheral acetylcholinesterase or may cause side effects. Thus, an alternative strategy consists in scavenging nerve agents in the bloodstream before they can reach acetylcholinesterase. Pre- or post-exposure administration of bioscavengers, enzymes that neutralize and detoxify organophosphorus molecules, is one of the major developments of new medical counter-measures. These enzymes act either as stoichiometric or catalytic bioscavengers.

Human butyrylcholinesterase is the leading stoichiometric bioscavenger. Current efforts are devoted to its mass production with care to pharmacokinetic properties of the final product for extended lifetime. Development of specific reactivators of phosphylated butyrylcholinesterase, or variants with spontaneous reactivation activity is also envisioned for rapid *in situ* regeneration of the scavenger.

Human paraoxonase 1 is the leading catalytic bioscavenger under development. Research efforts focus on improving its catalytic efficiency toward the most toxic isomers of nerve agents, by means of directed evolution-based strategies. Human prolidase appears to be another promising human enzyme. Other non-human efficient enzymes like bacterial phosphotriesterases or squid diisopropylfluorophosphatase are also considered though their intrinsic immunogenic properties remain challenging for use in humans. Encapsulation, PEGylation and other modifications are possible solutions to address this problem as well as that of their limited lifetime.

Finally, gene therapy for *in situ* generation and delivery of bioscavengers is for the far future, but its proof of concept has been established.

1. Introduction

The acute toxicity of organophosphorus compounds is due to the rapid phosphylation of acetylcholinesterase (AChE). Irreversible inhibition of AChE leads to an increase in acetylcholine concentration in the synaptic clefts and at neuromuscular junctions, disrupting the cholinergic neurotransmission. These organophosphorus inhibitors are phosphoryl/phosphonyl esters (Fig. 1). Thiono esters, e.g. parathion, need *in vivo* conversion into oxo forms to become cholinesterase inhibitors. While traditional organophosphorus nerve agents (OPNAs) pose the major threat, non-traditional nerve agents such as pesticides (malathion, paraoxon) or drugs (echothiophate) are potential terrorist threats. In addition, other organophosphorus compounds not specifically acting as inhibitors of AChE are potential threats too. For instance, TOCP (tri-o-cresyl phosphate), an anti-wear and flame retardant used in jet engine oils [1] is converted in the body to CBDP (Fig. 1), a potent inhibitor of carboxylesterases (CaEs), neuropathy target esterase and cholinesterases (ChEs) [2]. Also, the bicyclic phosphorus ester TMPP, formed during pyrolysis of synthetic engine oils, is

Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CaE, carboxylesterase; CBDP, 2-(o-cresyl)-4H-1,3,2-benzodioxaphosphoran-2-one or cresyl saligenin phosphate; ChE, cholinesterase; CHO, Chinese hamster ovary; HDL, high density lipoprotein; OPNA, organophosphorus nerve agent; PEG, polyethyleneglycol; hPON-1, human paraoxonase; PRAD, proline rich attachment domain; PTE, phosphotriesterase; TMPP, trimethylolpropane phosphate or ethyl bicylclophosphate; TOCP, triorthocresylphosphate.

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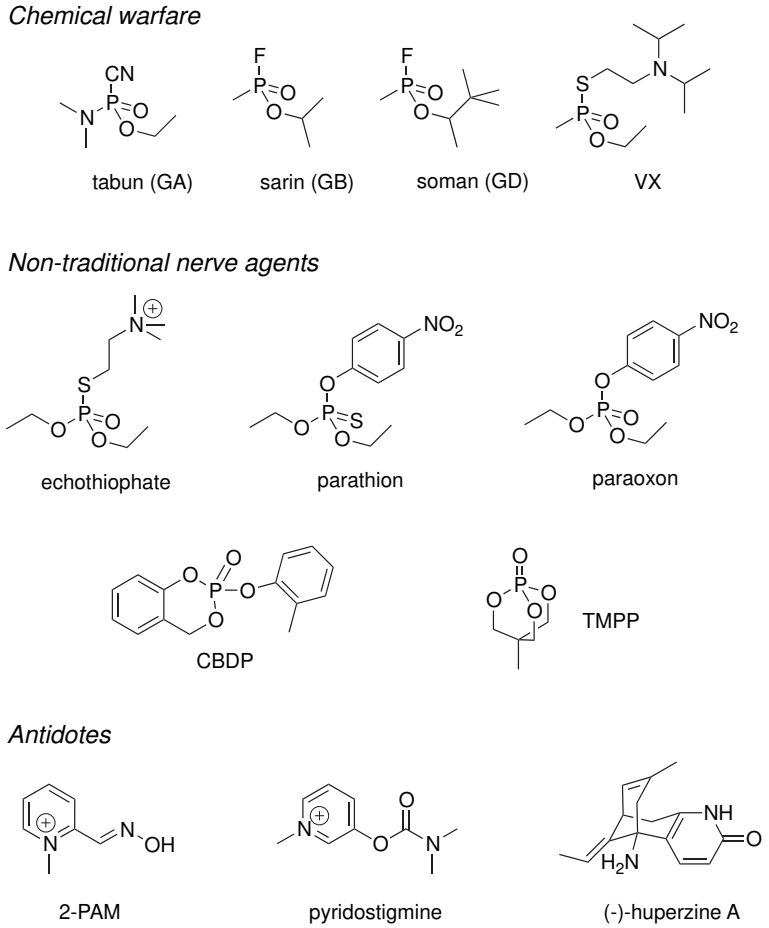

Fig. 1. Chemical structures of selected chemical warfare, non-traditional nerve agents, and antidotes.

a potent GABA antagonist. This compound, with a toxicity similar to that of sarin, has been called "the poor man's nerve agent" [3].

Medical treatments against OPNA poisoning aim at counter-acting the inhibition of AChE by the use of oxime reactivators, and prevent the primary and secondary effects of acetylcholine excess by the use of antinicotinic, antimuscarinic and anticonvulsant drugs. These approaches are more or less effective for mitigating the acute, sub-lethal and long-term effects of all agents. In the best case, they improve survival, but do not reduce incapacitation due to irreversible brain damage.

1.1. Current pre-treatment of OPNA poisoning

In situations where a risk of exposure to OPNAs is established, pre-treatments have to be implemented systematically for protection of personnel. Current prophylactic treatments consist in masking the active site of AChE from OPNA using specific ligands, which bind transiently to the enzyme (Fig. 2B). For example, this is achieved by the use of the carbamate pyridostigmine, a pseudo-irreversible inhibitor making a labile bond with the catalytic serine of AChE [4]. This strategy is effective only if a sufficient percentage of AChE is transiently inhibited by pyridostigmine, and remain hidden from the OPNA during the exposure time. Yet, pyridostigmine inhibition must remain moderate to keep a sufficient amount of active AChE to preserve cholinergic transmission before intoxication. It results that the balance between active/inhibited AChE can be challenging in terms of dosage. In addition, pyridostigmine does not readily cross the blood brain barrier [5], and leaves central AChE unprotected. In the search of a central AChE reversible inhibitor, (−)-huperzine A (Fig. 1), a natural alkaloid, appears to have the highest potential [6,7]. Nevertheless, it is toxic at those doses required for protection. Yet, a combination of (+)/(−) isomers of lower toxicity improves survival and reduces behavioral abnormalities against $1.2 \times LD_{50}$ of soman [8]. Also, recent huperzine A analogs were demonstrated to improve survival against $2 \times LD_{50}$ of soman in guinea pigs [9]. Other pharmacological pretreatments have been implemented in Western and other armies with known limitations and/or potent adverse effects (for review see Masson, 2011 and van Helden, 2011) [10,11].

1.2. Bioscavenger concept

The alternative approach to AChE inhibitors is based on molecules that inactivate OPNA in the bloodstream before they can reach AChE at the physiological sites. Bioscavengers being developed for some 25 years are stoichiometric, pseudo-catalytic, or catalytic. Stoichiometric bioscavengers are specific molecules that irreversibly bind to OPNAs in a mole-to-mole ratio (Fig. 2C). Pseudo-catalytic bioscavengers are stoichiometric bioscavengers in combination with a reactivator (Fig. 2D). Catalytic bioscavengers are OPNA-degrading enzymes with a turnover (Fig. 2E), so that administration of a small dose of a catalytic bioscavenger is thought to provide better protection than large doses of costly stoichiometric bioscavengers [12].

Numerous enzymes and proteins participate in natural defenses against OPNAs [13]. Natural skin, tissues and blood bioscavengers detoxify OPNAs or react with them. In some cases, these natural defenses are sufficient to protect against exposure to low doses

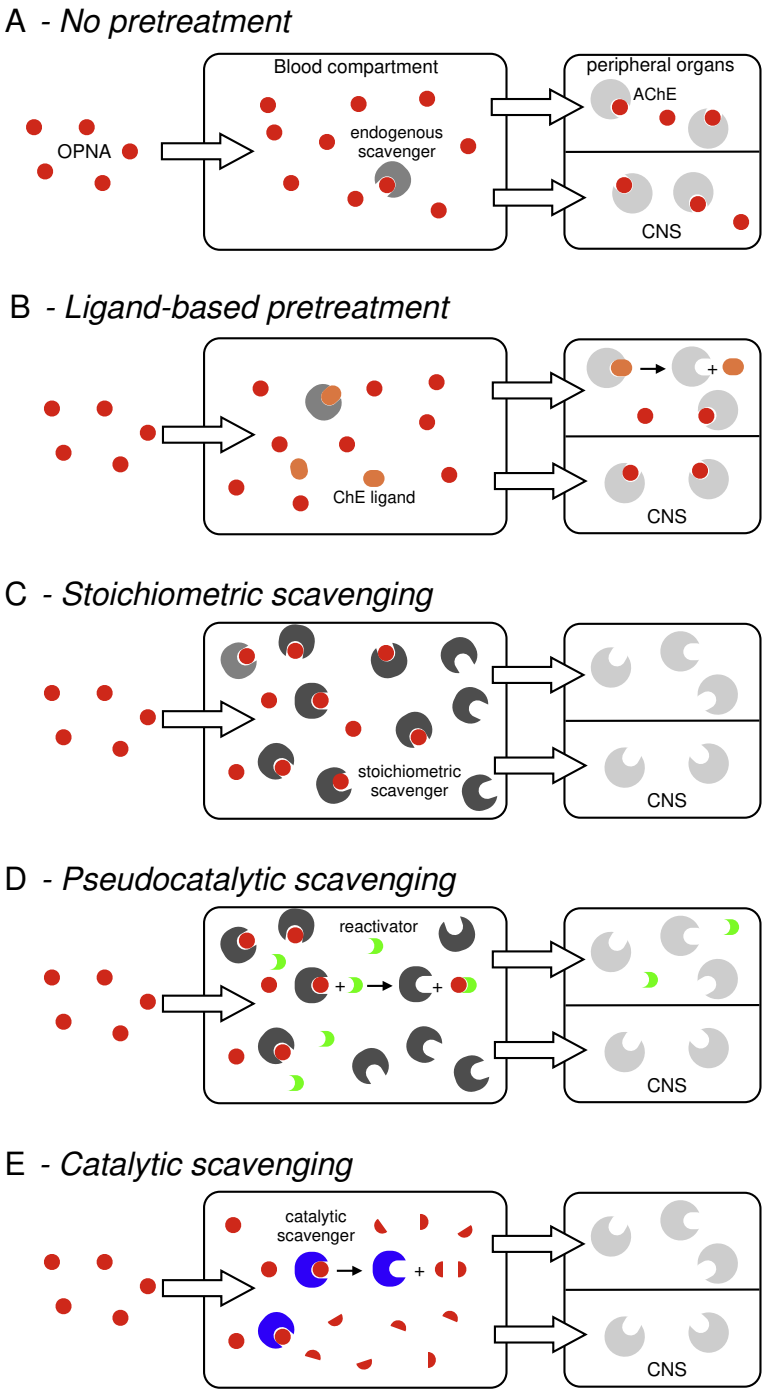

Fig. 2. Pretreatment strategies of OPNA poisoning. (A) OPNAs enter the blood compartment and pass to peripheral organs and the central nervous system to inhibit AChE. Endogenous scavengers like plasma BChE trap a small amount of OPNA. (B) The current pretreatment is based on a transient carbamate inhibitor, pyridostigmine, that masks the active site of a percentage of AChE from OPNAs. Active AChE is slowly released by spontaneous decarbamylation of the inhibited enzyme. Central nervous system AChE is not masked because pyridostigmine does not cross the blood–brain barrier. (C) Large amount of exogenous stoichiometric scavenger trap OPNAs in the blood preventing peripheral and central hAChE inhibition. (D) Co-administration of reactivators allows rapid regeneration of inhibited stoichiometric scavengers, thus potentially increasing the protection efficacy. (E) A limited amount of catalytic scavenger can hydrolyze every OPNA molecules in the blood compartment before they pass to peripheral organs and the central nervous system.

of OPNAs. For example, BChE (≈50 nM in human plasma) reacts rapidly with CBDP, the toxic metabolite of TOCP, and likely plays a role in protection against the development of the aerotoxic syndrome that may occur for low dose exposure to TOCP-containing fumes in aircrafts [14]. However, either they are abundant like albumin (≈0.6 mM in plasma and lymph) but react too slowly with toxicants, [15,16], or they are present in too low amount to confer protection against high doses of poisons. For example, CaEs present in plasma of model animals are effective endogenous bioscavengers [12], but human plasma is CaEs-free [17]. Protection in humans can be enhanced by administration of exogenous bioscavengers. Ideally, bioscavengers would protect against exposure to $5 \times LD_{50}$ of a large spectrum of OPNAs. Unlike pharmacological pretreatments, iatrogenic toxicity of bioscavengers should be negligible.

However, administration of large amounts of enzymes displaying promiscuous activities might perturb certain metabolic processes. An important issue is to administer bioscavengers devoid of protein contaminants that may cause side effects, e.g. endotoxins, coagulation factors. Thus, the medical use of bioscavengers imposes re-enforced GMP conditions for their preparation. Finally, we should point out that immunologic adverse effects are expected following repeated administration of non-human enzymes. PEGylation or inclusion of recombinant human and non-human enzymes and bacterial enzymes in nanocontainers should prevent immune responses. Recent reviews on the use of bioscavengers against OPNAs are in Romano et al., 2008 [18] and Gupta, 2009 [19]; see also, [20–22].

2. Stoichiometric scavengers

2.1. Prophylaxis

Human BChE (hBChE) is the most advanced bioscavenger. A dose of 200 mg of hBChE is predicted to protect a human against $2 \times LD_{50}$ of soman [23]. Animal studies showed that administration of large doses of hBChE confer protection against up to $5.5 \times LD_{50}$ of soman or $8 \times LD_{50}$ of VX [24]. Pre-treatment with 7.5 mg/kg completely prevent toxic signs and physiological abnormalities in minipig exposed for 1 h to sarin vapor (4.1 mg/m^3) [25]. The required doses to be injected in humans for such protection appear to be economically prohibitive, so that the current focus is on large-scale production. hBChE can be produced from different sources under GMP conditions. The most accessible natural source is Cohn fraction IV-4 paste, from which hBChE is purified with yields ranging around 7–9 g/100 kg [26]. Plasma-derived hBChE displays days of long-lasting stability in the bloodstream, and does not induce any side effect in rats [27]. Noteworthy, attempts to further extend the long half-life of plasma hBChE by PEGylation were not successful [28]. Behavioral and physiological safety of plasma hBChE was established at 30 mg/kg in rhesus monkeys [29] and 21 mg/kg in mice [30].

Albeit its great effectiveness, plasma hBChE presents the drawback that its source is limited, so that a maximum of 5000 individual doses could be theoretically produced yearly at the scale of a large country like the USA [31]. Therefore, production of recombinant hBChE (rhBChE) has been sought for the last decade. rhBChE has been produced in different expression systems including CHO cells [32], drosophila cells [33], silkworm larvae [34], but economically viable large-scale production are currently limited to transgenic goats [35] and tobacco plants [31,36]. Transgenic goat milk rhBChE used to be considered as the most promising production system, but a compromised lactation performance due to endoplasmic reticulum stress put a halt to development [37,38]. Purification of high-quality rhBChE can be costly due to limited performance of the key procainamide-based affinity chromatography step, but huprine-based resins improve yields and quality and should help to reduce global production costs [33].

Independent of the expression system, rhBChE has a dramatically shorter plasma half-life than the plasma enzyme (hours-long vs days-long) due to lower oligomerization and altered glycosylation pattern, especially incomplete sialylation [35,39,40]. Still, an extended biological life is achievable by simple addition [41] or coexpression [30,42] of a proline-rich peptide enhancing tetramerization such as PRAD, by fusion to albumin [43], chemical polysialylation [30] or PEGylation [35]. PEG-conjugated CHO-derived rhBChE has proved to be safe and not immunogenic [40]. To circumvent plasma half-life problems, aerosolized rhBChE can be delivered unmodified directly into the lung to form a shield against inhaled OPNAs. Such a non-invasive pretreatment 1–40 h prior intoxication by $>1 \times LD_{50}$ of aerosolized paraoxon can prevent inhibition of circulating cholinesterase in a dose-dependent manner [44].

An alternative to hBChE is the physiological target of OPNAs, i.e. human AChE (hAChE). hAChE is more stereoselective than hBChE, which is important in regard to the amount of enzyme required to scavenge one equivalent of racemic nerve agent. Indeed, half an equivalent of hBChE is sufficient if the enzyme binds preferably the same enantiomer as hAChE. At least one equivalent is necessary if hBChE binds equally both enantiomers or preferably the less toxic enantiomer. This last case is met for example with soman: the stereoselectivity ratio of hAChE for the P(S)-C(R)/P(R)-C(R) isomers of soman is 4×10^4-fold while hBChE has no selectivity [45]. Similar lack of strong selectivity of hBChE is observed for tabun [46] and V-agents [47], and indeed, at equivalent dose, hAChE provides a better protection of mouse exposed to VX compared to hBChE [48]. Yet, hAChE is not in advanced development as a stoichiometric bioscavenger, but mutants of the enzyme are being designed for pseudocatalytic scavenging (see Section 3).

2.2. Post-exposure treatment

Blood purification by hemodiafiltration was successfully implemented for a patient severely poisoned by sarin in the Tokyo subway [49]. However, the effectiveness of this approach is questionable, in particular in the case of percutaneous exposure (e.g. VX poisoning) and for OPNAs that store in lipophilic depot sites from which they are slowly released into the bloodstream.

By contrast a bioscavenger with a half-life matching or exceeding that of a nerve agent slowly absorbed via the percutaneous route, can effectively neutralize the agent as it continuously enters the blood compartment. In the case of VX, percutaneous absorption is sufficiently slow that i.m. injection of goat-milk rhBChE (220 mg/kg) 1 h after exposure to 2 and $5 \times LD_{50}$ improve survival rates of guinea pigs from 20% to 90% and 0% to 33%, respectively [50]. PEGylated goat-milk rhBChE (72 mg/kg) with improved residence time allows 100% survival of guinea pigs exposed to $2.5 \times LD_{50}$, with minimal signs of poisoning, despite a late i.m. administration 2 h after exposure [51]. Plasma-derived hBChE also provides 100% protection from lethality by identical conditions, but efficacy was shown to drop when administration was delayed at the onset of signs of poisoning (about 4–5 h) [52]. However, when classical therapy (oxime + anti-muscarinic + anticonvulsant) was concomitantly administered, a synergistic effect was observed as 100% survival was achieved, even for administration after the on-set of symptoms [53]. Synergy results from rapid alleviation of the cholinergic crisis by conventional treatment, while hBChE neutralizes further VX entering the bloodstream, thus preventing it from reaching the target organs.

3. Pseudocatalytic scavenging

The limit of a stoichiometric bioscavenger like hBChE is that once it is phosphylated by one molecule of OPNA, it becomes waste. An attractive approach is to recycle phosphylated hBChE by the mean of oxime reactivators. If the reactivation rates were in the same order as the inhibition rates, then a mixture of hBChE and reactivator would act as an effective pseudo-catalytic bioscavenger (Fig. 2D). Unfortunately, reactivation of hBChE by available oximes is too slow for pseudocatalytic scavenging of OPNA [54]. This led to seek better reactivators of hBChE [55]. Combinations of sub-stoichiometric amount of hBChE with last generations of specific hBChE reactivators show improved protective indices in therapy of sarin- and paraoxon-exposed mice [56].

Available oxime reactivators, having been primarily developed to reactivate hAChE, have shown to be more suitable for pseudoscavenging with hAChE than with hBChE. Actually, hAChE based pseudoscavenging has been early explored through one issue hampering the concept: phosphylated cholinesterases age, in a few minutes in the case of soman, and become refractory to oxime reaction [57]. F338A mutant of hAChE with slow aging rate, remaining reactivatable by oximes for extended periods, has enhanced pseudocatalytic properties [58,59]. This slow-aging mutation is effectively combined to the Y337A mutation aimed at enlarging the active site gorge for an easier access to reactivators [60].

4. Catalytic bioscavengers

Human enzymes capable of degrading OPNAs at high rate would be the most suitable biocatalytic scavengers. The idea of converting human ChEs into OPNA hydrolases was developed about 20 years ago. Several other human enzymes have been considered, including plasma paraoxonase-1 (PON-1), erythrocyte and liver prolidases, and human liver senescence marker (SMP-30) [61]. For a review on catalytic bioscavengers, see Wales et al. (2012) [62].

4.1. Cholinesterase-based catalytic scavengers

Mutagenesis of human cholinesterases into OPNA-degrading enzymes has been attempted. More than 60 mutants of hBChE and hAChE were created by inserting a second nucleophile in the active center (for reviews see: [63,64]). The Y124H/Y72D mutant of hAChE displays up to 110-fold improved spontaneous reactivation rate after inhibition by V-agents (half-life from 25 to 170 min), a performance not so far from the action of oxime reactivators [65]. Yet, this enzyme must be considered as a fast regenerating scavenger rather than a true catalytic scavenger. The G117H mutant of hBChE, that was the first designed ChE mutant capable of hydrolyzing OPNAs, remains the most active of all, but still far too low for practical interest as a catalytic scavenger. Interestingly, transgenic mice expressing G117H hBChE are protected against $1 \times LD_{50}$ echothiophate toxicity not because of echothiophate neutralization, but most likely because G117H hBChE is more slowly inhibited, readily regenerates and finally acts as a surrogate of wild-type mouse AChE and BChE [66]. Besides, G117H/E197Q hBChE, a slow aging/fast regenerating variant expressed in transgenic plants, failed to protect guinea pigs from $2 \times LD_{50}$ challenges of GB/GD/VX, due to 1000-fold decreased phosphylation rates for this particular mutant [31]. Since the X-ray structure has recently been solved [67], there is renewal of effort to improve the dephosphylation rates of the G117H mutant without altering phosphylation rates, its mechanism being now investigated by QM/MM modeling [68,69]. It is believed that these works will lay foundations for future computational re-design of a new generation of hBChE mutants aimed at hydrolyzing OPNAs (Lushchekina et al., unpublished work).

4.2. Human paraoxonase 1

A considerable body of data is available on human paraoxonase 1 (hPON-1) as a potential catalytic bioscavenger (for a review see: [70]). hPON-1 is a calcium dependent promiscuous enzyme (lactonase, arylesterase, phosphotriesterase). Promiscuous activities are controlled by position of the catalytic Ca^{2+} in the catalytic center [71]. hPON-1 is produced in the liver, and circulating in the blood bound to HDLs. Expression and plasma concentration can be positively modulated by hypolipemic drugs and polyphenols [72]. hPON-1 hydrolyzes numerous OPNAs at quite a high rate [21]. hPON-1 level is inversely correlated with susceptibility to OPNAs intoxication. For example, *Trichoplusia ni* larvae expressing hPON-1 are protected from exposure to $>100 \times LD_{50}$ of chlorpyrifos [73]. Conversely, KO-mice for hPON-1 are far more sensitive to OPNAs than wild-type mice [74]. Though hPON-1 hydrolyzes preferentially the less toxic soman enantiomers [75], a recent *in vitro* study using hAChE back-titration showed that tabun is efficiently hydrolyzed [76]. In addition, intravenous administration of purified hPON-1 was found to protect guinea pigs against sarin and soman [77,78]. However, exogenous injection of hPON-1 produced in *Trichoplusia ni* larvae or adenovirus-induced expression of hPON-1 do not sufficiently increase the systemic activity in mice to provide *in vivo* protection against nerve agents [79].

A 10- to 100-fold increase in hPON-1's catalytic efficiency (k_{cat}/K_m) would be enough to afford protection against nerve agents. Modeling OPNA interactions in a hPON-1's 3D structure model [80] helps to design new mutants of the enzyme by the site-directed mutagenesis approach [81,82]. H115W/Y71A double mutant improve paraoxon hydrolysis rate 8-fold over wild-type, but gains for VX, GA, GB and GD remain below 2-fold [83]. Computer-assisted redesign of hPON-1 mutants with enhanced hydrolase activity and better enantioselectivity would need exact knowledge of the 3D structure and molecular dynamics of the human enzyme, which are still lacking. Alternatively, directed evolution of a chimeric PON-1 (chimPON-1) made via mammalian gene shuffling, combined with high-throughput screening, successfully led in 5 generations to an evolved variant of chimPON-1 that hydrolyzes the most toxic enantiomer of a coumarin analog of cyclosarin with a catalytic enhancement (k_{cat}/K_m) of 100,000 [84,85]. This variant protects mice challenged with $1 \times LD_{100}$ cyclosarin analog [84]. Unfortunately, chimPON-1 is expected to be immunogenic, and the catalytic properties of variants are not easily transposable to hPON-1 [81].

Plasma-derived hPON-1 is difficult to purify and its stability depends on the presence of partners and a hydrophobic environment [65,86]. Therefore, its mass production from outdated plasma would present numerous biotechnological problems. Recombinant hPON-1 and mutants are functionally expressed with difficulties in mammalian cells, *Escherichia coli* [87], and insect (*Trichoplusia ni*) larvae [73]. To overcome biotechnology difficulties with production, stabilization and pharmacokinetics of a functional biopharmaceutical, gene delivery of hPON-1 has been attempted. A recent study using an adenoviral vector demonstrates that endogenous production of high levels of hPON-1 is capable of protecting mice against cumulative doses ($4 \times LD_{50}$) of diazoxon [88]. It is therefore quite conceivable that a safe gene-delivered evolved hPON-1 variant would be able to provide excellent protection against OPNAs. Thus, hPON-1 remains the most promising catalytic bioscavenger.

4.3. Other phosphotriesterases

Other phosphotriesterases (PTEs) have been considered as potential catalytic bioscavengers. The squid (*Loligo vulgaris*) diisopropyl fluorophosphatase with a β-propeller structure similar to hPON-1, hydrolyzes OPNAs [89]. Rational design allowed to reverse the stereochemical preference of this enzyme for the most toxic OPNA isomers [90]. A PEGylated form of the mutant with improved stereoselectivity prevents death of rats exposed to a subcutaneous $3 \times LD_{50}$ dose of soman [91].

Several bacterial PTEs, mesophilic and extremophilic, have been extensively investigated for some 20 years (for reviews see [92,93]), but screening of new bacterial strains for measurable level of OPNA hydrolase activity is still ongoing [94]. Strategies combining rational design and directed evolution have been extremely

successful at improving native phosphotriesterase activity. Such strategies led to the discovery of the H257Y/L303T mutant of *Pseudomonas diminuta* PTE, with up to 15000-fold improvements in catalytic activity against the most toxic enantiomers of GB, GD and GF, thus reaching 1.2×10^8, 3×10^7 and 5×10^7 M^{-1} min^{-1}, respectively [95]. The catalytic activity of a phosphotriesterase from *Deinicoccus radiodurans* toward ethyl and methyl paraoxon was improved respectively 560 fold and 180-fold to reach k_{cat}/K_M about 4.6×10^5 M^{-1} min^{-1} and 6.3×10^3 M^{-1} min^{-1}[96].

Despites these successes, important issues remain before PTE can be used *in vivo* for OPNA detoxification, notably issues about expression, pharmacokinetics and humanization. Indeed, despite their bacterial origin, large scale-production of PTEs in *E. coli* is problematic due to limited synthesis of the bimetal center [97,98]. Regarding pharmacokinetics, PTE from *Agrobacterium radiobacter* prevents lethality of rat treated with pesticides [99], but has a relatively short mean residence time in primates (57 min) [100]. PEGylation of PTE improves thermal stability [101] and extends mean residence time from a few minutes to a few days in rats, but the modified enzyme remains immunogenic, thus of limited use in humans [65].

4.4. Other human and mammalian enzymes

To avoid immunogenicity issues, research groups were led to look for any human protein that has the potential to form the basis of a pretreatment. The drug-metabolizing human liver enzyme carboxylesterase 1 (hCaE1) was found to be a candidate of interest. Wild-type hCaE1 reacts with G-agents and reactivates spontaneously after inhibition by the P-(S) isomer of sarin at a slow rate (half-time = 46 h) [102]. The rate is enhanced by the addition of an oxime according to the pseudocatalytic bioscavenger concept. Like for cholinesterases, the introduction of nucleophilic residues near the triad can enhance the spontaneous reactivation rate up to a half time of 1 h in the case of cyclosarin [103]. But like cholinesterase, the gain is too modest for catalytic scavenging.

Human prolidases from erythrocyte or liver present structural and catalytic properties similar to *Alteromona's* "organophosphorus acid anhydrolase", aka phosphotriesterase [104,105]. Human prolidases are easily expressed in *E. coli* [106] and were found to hydrolyze the G-agents at a higher rate than hPON-1. Prolidase can hydrolyze tabun but not CBDP nor TMPP [14]. Gene-delivered prolidase persistenly expressed in mice, provides only modest protection *in vivo* delaying death by only 4–8 h [107]. However, engineering of this enzyme is just beginning.

The senescence marker protein 30 is another human enzyme of interest [108]. It is a promiscuous metal-containing enzyme lactonase structurally related to hPON-1 and DFPase [109]. Its phosphotriesterase activity is against G-agents, DFP and paraoxon, albeit at modest rates for the mouse homologous enzyme [61]. As for human prolidase, study of this enzyme is still in infancy.

Another mammalian enzyme, the zinc-containing mouse adenosine deaminase, was redesigned for organophosphate hydrolysis [110]. After directed evolution of the initial redesign, the catalytic efficiency (k_{cat}/K_M) raised up to $\sim 10^4$ M^{-1} s^{-1} for the hydrolysis of a model organophosphate, a value just one order shy of the efficiency required for a catalytic bioscavenger (10^5 M^{-1} s^{-1}) [63].

5. Future directions

Human BChE is currently the most advanced bioscavenger, but an affordable source of the enzyme is still lacking. Production in transgenic organisms remains the only viable long-term solution, in spite of the complications encountered with transgenic goats. Other animals, like transgenic rabbits are under evaluation, and production in the leaves of tobacco plants gives promising yields [31]. New purification protocols improving yields and quality will also help to reduce production costs [33]. But recombinant hBChE lacks the post-translational modifications necessary to achieve daylong residence time, in particular glycosylation and tetramerization. Solutions for tetramerization already exist, so that the most serious issues revolve around the glycosylation pattern of recombinant hBChE. Chemical modifications like PEGylation or polysialylation [30] extend residence time to a sufficient level, but their cost for hundred grams of enzyme is probably prohibitive. Enzymatic polysialylation by the mean of bacterial sialyltransferases is potentially more affordable [111]. Modification of transgenic hosts to optimize *in vivo* human-like glycosylation with polysialylation during biosynthesis could solve the issue. Though, in the case of the humanization of plants, the endogenous glycosylation pathway must eventually be partially or completely shut down to avoid mixed plant/human patterns. Consequences of such extreme modifications on the plant viability are unclear yet.

Alternatively to glycosylation, encapsulation in nanocarriers lengthens residence time to multiple days: hBChE encapsulated in polylysine/polyethylene oxide copolymers, injected i.m. or i.v. in mice, is capable of crossing the blood brain barrier and remains active for 3 days in the brain [112]. Encapsulation of recombinant and/or non-human enzymes may also offer the advantage of cheating the immune system.

Delivery of protein scavenger in human is also challenging given the mg-dose required for effective protection. In addition to i.v. injection (not compatible with field use) the privileged delivery route is i.m. injection. Yet, very few is known about diffusion of protein bioscavengers following i.m. injection in humans. Related pharmacodynamic/pharmacokinetic models are still lacking. Alternative delivery means must be evaluated in addition to aerosols [44], e.g. intranasal delivery [113] or transdermal means like microneedle patches.

The use of safe and short-induction gene vectors capable of delivering hBChE in the bloodstream would solve both the delivery and cost issues. The excellent protection afforded by gene-delivered hBChE, using an adenoviral system in mice challenged with OPNAs ($5 \times LD_{50}$ VX, $30 \times LD_{50}$ echothiophate) proved the feasibility of this approach [114]. Similar success was recorded for hPON-1 [88] and prolidase [107]. However, before gene delivery becomes operational, further work is needed to engineer safe vectors that do not produce toxic viral proteins and/or induce deleterious immune responses. Also, the use of a gene for pretreatment of healthy human beings poses a delicate ethical issue, regardless of the transient nature of the expression.

With regard to catalytic bioscavengers, human prolidase is a recent candidate that will be further studied. Mutants of rPON-1, bacterial phosphotriesterase with good catalytic efficiencies against the toxic isomers of OPNAs are now identified. But these enzymes are not human enzymes and must be humanized. The directed-evolution work performed on rPON-1 is not transposable to hPON-1 [81]. In that context, the determination of the 3D structure of hPON-1 remains a priority to improve its catalytic efficiency towards nerve agents.

In addition, pharmaceutical biotechnology of hPON-1 is complex. The enzyme is unstable without a proper hydrophobic environment. Binding of engineered hPON-1 to artificial HDL (Tawfik et al., personal communication) appears to be a solution for the future.

Research of novel OPNA-degrading enzymes is actively pursued [94], in particular in extremophile microorganisms [115]. Evolved mutants of *Sulfolobus solfataricus* PTE have been found to degrade nerve agent analogues and CBDP at very fast rate ($k_{cat}/K_m \approx 10^7$–10^8 M^{-1} min^{-1}) [14,116]. In addition, mass production in *E. coli*, and easy purification of these enzymes seems affordable.

Given the current limited activity spectrum of OPNA-reacting enzymes, operational catalytic bioscavengers should be cocktails of different enzymes.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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