Chemico-Biological Interactions 292 (2018) 50–64

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Catalytic bioscavengers as countermeasures against organophosphate nerve agents

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ARTICLE INFO

Keywords:
Organophosphates
Nerve agents
Bioscavengers
Intoxication
Enzymes
Medical countermeasures

ABSTRACT

Recent years have seen an increasing number of incidence, in which organophosphate nerve agents (OPNAs) have been used against civilians with devastating outcomes. Current medical countermeasures against OPNA intoxications are aimed at mitigating their symptoms, but are unable to effectively prevent them. In addition, they may fail to prevent the onset of a cholinergic crisis in the brain and its secondary toxic manifestations. The need for improved medical countermeasures has led to the development of bioscavengers; proteins and enzymes that may prevent intoxication by binding and inactivating OPNAs before they can reach their target organs. Non-catalytic bioscavengers such as butyrylcholinesterase, can rapidly bind OPNA molecules in a stoichiometric and irreversible manner, but require the administration of large protein doses to prevent intoxication. Thus, many efforts have been made to develop catalytic bioscavengers that could rapidly detoxify OPNAs without being inactivated in the process. Such enzymes may provide effective prophylactic protection and improve post-exposure treatments using much lower protein doses. Here we review attempts to develop catalytic bioscavengers using molecular biology, directed evolution and enzyme engineering techniques; and natural or computationally designed enzymes. These include both stoichiometric scavengers and enzymes that can hydrolyze OPNAs with low catalytic efficiencies. We discuss the catalytic parameters of evolved and engineered enzymes and the results of *in-vivo* protection and post-exposure experiments performed using OPNAs and bioscavengers. Finally, we briefly address some of the challenges that need to be met in order to transition these enzymes into clinically approved drugs.

1. Introduction

Current medical countermeasures for treating organophosphate nerve agent (OPNA) intoxication include: atropine, oxime reactivators, and anticonvulsants. If applied in time, they can prevent lethality and mitigate the intoxication symptoms. However, they may fail to prevent a cholinergic crisis that would lead to loss of consciousness and permanent brain damage [1–3]. In addition, they are not suitable as preventive measures prior to intoxication, as these drugs produce severe side effects such as: CNS impairment, increased blood pressure and increased heart rate if administered prior to an OPNA intoxication [4]. In fact, apart from the stoichiometric bioscavenger human butyrylcholinesterase (HuBChE), purified from human blood, there is no available prophylactic treatment that can prevent OPNA intoxication and the onset of its symptoms. In recent years, catalytic bioscavengers have been proposed as the next generation of medical countermeasures that may allow efficient prophylactic protection from high doses of OPNAs using small doses of protein [5,6].

Catalytic bioscavengers are enzymes that can detoxify OPNAs by performing multiple cycles of OPNA binding and hydrolysis. They have a clear advantage over stoichiometric ones since unlike the latter, which inactivate OPNAs by binding to them irreversibly, their interaction with OPNAs results in reversible binding and rapid hydrolysis of the OPNAs. In principle, this should enable small amounts of catalytic scavenger to detoxify lethal doses of nerve agents *in-vivo* before the latter can inactivate acetylcholinesterase (AChE) at important physiological sites; and to afford protection from multiple OPNA exposures without being consumed. In contrast, the high molecular weight of stoichiometric scavengers such as butyrylcholinesterase (BChE) or AChE and the requirement for a one-to-one ratio of non-catalytic scavenger to OPNA molecule, imply that large protein doses (i.e. hundreds of mgs) are required to provide protection from OPNA intoxication using stoichiometric scavengers [7,8]. Use of high protein doses of BChE or AChE for protection can be costly [9], and may also increase the chances of adverse physiological reactions following their administration [10]. Thus, during the past decade an increasing number of

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https://doi.org/10.1016/j.cbi.2018.07.006
Received 20 May 2018; Received in revised form 4 July 2018; Accepted 6 July 2018
Available online 07 July 2018
0009-2797/

efforts have been devoted to the development of catalytic bioscavengers [5,6].

Pseudo-catalytic bioscavengers are enzymes or combinations of enzymes and chemical reactivators that can jointly perform multiple cycles of binding and hydrolysis of organophosphates. The term usually refers to stoichiometric scavengers from the family of B-*esterases,* such as AChE, BChE or carboxylesterase (CaE) [11], complexed with an oxime reactivator in their active site. Following the interaction of the enzyme with an OP inhibitor, it may undergo two consecutive reactions: First, covalent binding of the OP to its catalytic serine residue. Second, hydrolysis and release of the inactivating OP [12]. The rates of spontaneous detachment of the inhibitor from the catalytic serine depend on the type of enzyme and OP, but are usually very slow for B-esterases interacting with OPNAs. Therefore, external nucleophiles (i.e. chemical reactivators) are required to turn B-esterases into pseudo-catalytic OP hydrolyzing systems [13–15].

There are several advantages to pseudo-catalytic bioscavengers: First, their protein component is normally present in the circulation (e.g. BChE [16] in human serum and AChE [17] on red blood cells). Second, they rapidly sequester all types of OPNAs [18] as the latter are designed as cholinesterase inhibitors in the first place. Lastly, their reactivating oximes can reach inhibited AChE in peripheral nerve tissues and in some cases, in the central nervous system (CNS) [19–21]. However, there are several disadvantages to currently available reactivators: They exhibit narrow OPNA specificities and enable effective reactivation of only a number of OPNAs per reactivator [22,23]. They become ineffective following aging of the OP-cholinesterase bond, which is a rapid process in the case of OPNAs such as soman [24]. They have short circulatory residence times, and need replenishing in cases of continuing OPNA exposures [14]. Finally, the rate of OPNA hydrolysis by oxime-mediated reactivation, even by the most effective combinations of oxime, cholinesterase and OP (e.g. cyclosarin-inhibited HuAChE reactivated with HLö7, $k_{r2} = 9.3 \times 10^4$ [$M^{-1}min^{-1}$] [25]), is ~2–3 orders of magnitude slower than the hydrolytic rate required to efficiently prevent intoxication (see bellow). Therefore, the current status of pseudo-catalytic scavengers, suggests they are not efficient enough to detoxify OPNAs and prevent the inhibition of AChE at peripheral and CNS sites.

A more effective way of preventing OPNA intoxication would be to employ a highly efficient, broad-spectrum catalytic bioscavenger, which could rapidly inactivate the toxic isomers of OPNAs in the circulation. In order to do so using a low enzyme treatment-dose (< 1 mg/kg), theoretical models predict that catalytic bioscavengers must have a catalytic efficiency (k_{cat}/K_M) of $\geq 1 \times 10^7$ [M^{-1} min^{-1}] with the toxic isomers of OPNAs [26,27]. Using enzymes with higher catalytic efficiencies (i.e. (k_{cat}/K_M) of $\geq 5 \times 10^7$ [M^{-1} min^{-1}]) as prophylactics would detoxify 96% of the OP in the circulation in less than 10 s and provide sign-free protection from intoxication [5,28]. Unfortunately, OPNAs are xenobiotic compounds that serve as promiscuous substrates for natural OP hydrolyzing (OPH) enzymes and are hydrolyzed with low catalytic efficiencies [29,30]. In addition, most OPH enzymes isolated so far tend to bind and inactivate the less toxic isomers of OPNAs more efficiently than the toxic ones [31,32]. Therefore, in order to obtain catalytic bioscavengers that could be used as effective medical countermeasures for OPNA intoxication, there is a need to greatly enhance both the activity and selectivity of natural enzymes towards the toxic isomers of OPNAs. In recent years, directed evolution and protein engineering techniques have been used successfully to generate catalytic bioscavengers with such properties [33–38]. Here we aim to discuss proteins that have been suggested as candidates for catalytic bioscavenging, their activities and the efforts made to increase their OPNA hydrolyzing capabilities.

2. Turning stoichiometric bioscavengers into catalytic ones

The antidotal and therapeutic potentials of a human B-family esterase, endowed with a high turnover rate for OPNA binding and hydrolysis, are great. Such a bioscavenger would have all the advantages of a stoichiometric scavenger (e.g. high OPNA binding rates, long circulatory residence times, biocompatibility), without the loss of activity that follows its interaction with OPs. This has prompted many attempts to design or engineer B-esterase variants that could perform multiple turnovers with OPNAs, alone, or in combination with specific reactivators.

2.1. Carboxylesterases

Carboxylesterases (CaEs) are a ubiquitous family of esterases (EC 3.1.1.1) that hydrolyze structurally diverse carboxylic esters to their corresponding alcohols and carboxylic acids [39,40]. Their activity, *in-vivo*, can either activate or inactivate compounds, but since their endogenous substrates are unknown, they are considered to play a protective physiological role by detoxifying xenobiotics [40]. Of the 5 human isoforms of CaE, only two have been extensively studied: Human carboxyl esterase 1 (HuCaE1) that is present mostly in human liver, macrophages, and lung epithelia and human intestinal carboxylesterase (HuiCaE) that is expressed especially in the small intestine, kidney, heart and skeletal muscle [41]. HuCaE1 is a 60 kDa protein that possess a much larger active-site pocket relative to HuAChE and can bind larger substrates [41]. It catalyzes ester hydrolysis using a catalytic triad (Ser221, Glu353, His 464) and was suggested to have a “side door” to facilitate the exit of its hydrolysis products [42]. CaEs lack a catalytic site tryptophan residue that is conserved in the cholinesterase family (e.g. Trp 86 in HuAChE), and so the enzyme reacts slowly with charged OPs and oxime reactivators.

The abilities of CaEs to bind and detoxify OPNAs and pesticides were investigated primarily using rodent CaEs such as mouse or rat plasma carboxylesterase (CES1C) [43–45]. CES1C binds uncharged OPNAs such as sarin and soman, and pesticides such as paraoxon and DFP more rapidly than charged OPs such as VX. Both HuCaE and CES1C do not undergo aging regardless of the structure of the inactivating OP [44,46]. HuCaE1 is completely inhibited by soman and cyclosarin and undergoes very slow, spontaneous reactivation following inhibition by sarin with a half-time of 45 h [46]. Attempts to increase the reactivation rates of OPNA-inhibited CaEs using oxime nucleophiles, revealed that they react poorly with charged oxime compounds and much better with uncharged ones [46,47]. However, even in the presence of uncharged oximes, the half-life time for reactivation of HuCaE1 ($t_{1/2}$) was too slow to promote rapid catalytic detoxification (e.g. the $t_{1/2}$ of serine-inhibited HuCaE1 in the presence of 2,3-butanedione monoxime is 41 min [46]).

Investigations of insect resistance to OP pesticides have revealed that while some insects overproduce esterases, such as CaE, to elicit resistance by sequestering the intoxicating OP [48], others have evolved mutants of CaE that are capable of hydrolyzing the pesticides [49,50]. In the case of the E3 CaE isozyme from *L. cuprina*, a single active site mutation, G137D, was sufficient to confer OP resistance by acting as a general base catalyst that can hydrolyze the OP-bound enzyme [50,51]. To enhance the OPNA hydrolase activities of HuCaE1, two active-site amino-acid substitutions were rationally designed based on the crystal structure of the enzyme. They were aimed to facilitate the reactivation of the OP-bound catalytic Ser 221 residue via the positioning of a water molecule in its vicinity [52]. Indeed, the V146H/L363E HuCaE1 mutant replaced two hydrophobic residues on opposite sides of the active site pocket with charged residues that increased the reactivation rates of the enzyme without reducing its affinity to the OPNAs [52]. As a result, the reactivation rates of sarin, soman, and cyclosarin-inhibited HuCaE1 increased by 5-, 20-, and ~33,000-fold and their half-life times decreased to 9.5 h, 11.5 h and 1.2 h respectively [52]. This CaE mutant was significantly improved in reactivation rates relative to the wt protein, and was able to hydrolyze the toxic isomer of a cyclosarin analog at a rate of 5.3×10^4 [M^{-1} min^{-1}]. However, this