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Prüfung der Wirksamkeit chemischer Scavenger als Therapeutika bei Organophosphatvergiftungen

Examination of effectiveness of chemical scavengers as therapeutics in organophosphate poisoning

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Abstract

Cholinergic crisis triggered by inhibition of cholinesterases *via* organophosphorus nerve agents (OP) and pesticides is treated with atropine and a reactivator of inhibited cholinesterase, called oxime. Multiple *in vitro* and *in vivo* studies demonstrated that this standard therapy may secure survival, but is insufficient in preventing incapacitation and lacks efficacy against several nerve agents and pesticides. Over the years, novel therapy approaches have been closely investigated with promising candidates being non-oxime reactivators and scavengers based on enzymes (bioscavengers) or small molecules. The low efficacy and immunological compatibility are main disadvantages of bioscavengers, thus the focus of the presented thesis is on a small molecule scavenger and a non-oxime reactivator.

Detoxification of OP by cyclodextrins (CD), a macrocycle, was recognized early and optimized by inserting a nucleophilic group at the rim of the CD cavity. But the development of a more potent scavenger with a broad spectrum activity is closely linked to gathering information about inclusion complexes in cyclodextrins and their influencing factors. For that reason a structure-activity study with a 6-pyridinium oximate substituted β-cyclodextrin and alkyl methylphosphonofluoridates (sarin derivatives) was performed. Therefore, it can be concluded that the oxime substituted β-cyclodextrin better detoxifies sarin derivatives than native β-cyclodextrin, conforming involvement of the nucleophilic group in OP degradation. Good inclusion of OP in the CD cavity is correlated with a higher stability constant and improved degradation. Data imply efficient detoxification of alkyl methylphosphonofluoridates, thus the oxime substituent β-cyclodextrin is a promising small molecule scavenger for poisoning with G-type nerve agents.

Another novel approach for the treatment of OP poisoning could be the use of small molecules as non-oxime reactivators. The antimalarial drug amodiaquine was identified as a promising candidate without further investigating its potential against a broad range of OP. Therefore amodiaquine's interaction with human acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in presence or absence of OP was investigated and revealed a reversible inhibition of cholinesterases by amodiaquine and a mixed competitive-non-competitive inhibition type with AChE. Amodiaquine inhibited AChE more potently than BChE, wherefore a dose escalation was not possible. Reactivation of cyclosarin-, sarin-, and VX-inhibited cholinesterases by amodiaquine occurred, whereas reactivation of tabun-inhibited cholinesterase was insufficient. In a dynamic model, amodiaquine was administered prior to soman poisoning, but no significant beneficial effect on cholinesterases or reactivation of inhibited cholinesterases was observed. However, amodiaquine's mode of action in reactivating OP-inhibited cholinesterases is not fully understood, but research efforts should continue in order to generate a more potent non-oxime reactivator. In summary, small molecules as scavengers and non-oxime reactivators have a promising potential as novel therapeutics for the treatment of OP poisoning. Still, further investigations are necessary and should progress on existing experiences.

Zusammenfassung

Eine cholinerge Krise, die durch Cholinesterasehemmung auf Grund einer Vergiftung mit phosphororganischen Nervenkampfstoffen (OP) und Pestiziden ausgelöst wird, wird mit Atropin, und einem Cholinesterase-Reaktivator, welcher als Oxim bezeichnet wird, behandelt. Viele *in vitro* und *in vivo* Studien bestätigen, dass diese Standardtherapie das Überleben sichern kann, aber nur unzureichend vor den Vergiftungssymptomen schützt und unwirksam bei einigen Nervenkampfstoffen und Pestiziden ist. In den letzten Jahren wurden neue Therapieansätze eingehend untersucht, wobei Nicht-Oxim Reaktivatoren und Scavenger, basierend auf Enzymen (Bioscavenger) oder kleinen Molekülen („small molecule scavenger“) aussichtsreiche Kandidaten sind. Die geringe Wirksamkeit und immunologische Verträglichkeit sind die größten Nachteile von Bioscavengern, weshalb der Fokus in der vorliegenden Arbeit auf einen small molecule scavenger und ein Nicht-Oxim Reaktivator liegt.
Frühzeitig wurde OP-Bindung und -Abbau durch Cyclodextrine (CD), einem Makrozyklus, erkannt und durch das Einfügen einer nukleophilen Gruppe am Rand des CD-Hohlraumes optimiert. Aber die Entwicklung von einem hoch wirksamen Scavenger mit einem breiten Wirkungsspektrum ist eng mit der Gewinnung von Informationen über Einschlusskomplexe in CD und deren Einflussfaktoren verbunden. Aus diesem Grund wurde eine Struktur-Aktivitätsuntersuchung mit einem 6-Pyridiniumoximat substituiertem β-CD und Alkylmethylfluorphosphonaten (Sarin-Derivate) durchgeführt. Daraus ergab sich, dass das Oxim-substituierte β-CD die Sarin-Derivate besser entgiftete als das native β-CD, wodurch die Beteiligung der nukleophilen Gruppe am OP-Abbau bestätigt wurde. Ein guter Einschluss des OP in den CD-Hohlraum korrelierte mit einer hohen Stabilitätskonstante und verbessertem Abbau. Die Daten zeigen eine effiziente Entgiftung von Sarin-Derivaten an und dass das Oxim-substituierte β-CD ein vielversprechender small molecule scavenger bei Vergiftungen mit G-Kampfstoffen sein könnte.
Ein weiterer therapeutischer Ansatz bei OP-Vergiftungen kann der Einsatz von kleinen Molekülen als sogenannte Nicht-Oxim Reaktivatoren sein. Das Antimalaria-Medikament Amodiaquin wurde als ein vielversprechender Kandidat identifiziert, ohne dessen Potenzial gegenüber einem breiten Spektrum von OP weiter zu untersuchen. Daher wurden Interaktionen zwischen Amodiaquin mit humaner Acetylcholinesterase (AChE) und Butyrylcholinesterase (BChE) in Gegenwart und Abwesenheit von Nervenkampfstoffen untersucht und dabei festgestellt, dass Amodiaquin Cholinesterasen reversibel hemmt und für die AChE wurde ein gemischter kompetitiv-nicht-kompetitiver Hemmtyp identifiziert. Amodiaquin hemmte AChE stärker als BChE, weshalb eine Dosissteigerung nicht möglich war. Reaktivierung von Cyclosarin-, Sarin- und VX-gehemmter Cholinesterase durch Amodiaquin trat auf, wohingegen die Reaktivierung von Tabun-gehemmter Cholinesterase unzureichend war. In einem dynamischen Modell wurde Amodiaquin vor einer Somanvergiftung verabreicht, jedoch konnte kein deutlicher Schutzeffekt mit Cholinesterasen oder Reaktivierung von gehemmter Cholinesterase beobachtet werden. Obwohl der Wirkmechanismus von Amodiaquin bei der Reaktivierung von OP-gehemmten Cholinesterasen nicht vollständig verstanden wurde, sollte weiter geforscht werden, damit ein wirksamerer und weniger toxischer Nicht-Oxim Reaktivator gefunden werden kann. Zusammenfassend stellen kleine Moleküle als Scavenger und Nicht-Oxim Reaktivatoren einen potenziell neuen Therapieansatz für die Behandlungen von OP-Vergiftungen dar. Dennoch sind weitere Untersuchungen nötig und sollten auf gewonnenen Erfahrungen aufbauen.

Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema „Prüfung der Wirksamkeit chemischer Scavenger als Therapeutika bei Organophosphatvergiftungen“ selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, den 20.07.2016

(Anne Bierwisch)

Contents

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Abbreviations

AChE Acetylcholinesterase

ADOC 4-amino-2-((diethylamino)methyl)phenol

BChE Butyrylcholinesterase

CD Cyclodextrin

DFP Diisopropyl fluorophosphate

DNA Deoxyribonucleic acid

e. g. *Exempli gratia*; for example

GA Tabun; Ethyl-*N,N*-dimethylphosphoramidocyanidate

GB Sarin; *O*-Isopropyl methylphosphonofluoridate

GD Soman; Pinacolyl methylphosphonofluoridate

GF Cyclosarin; Cyclohexyl methylphosphonofluoridate

HI-6 1-[[[2-[(hydroxyimino)methyl]pyridinium-1-yl]methoxy]methyl]-pyridinium dichloride

IC_{50} Half maximal inhibitory concentration

k_{cat} Unimolecular rate constant

k_{detox} Second-order detoxification constant

K_M Michaelis constant

LD_{50} Median lethal dose

µM Micromolar

mg Milligrams

OP Organophosphorus nerve agents

2-PAM 2-Pyridine aldoxime methyl chloride; Pralidoxime

PTE Phosphotriesterase

PXE Paraoxon-ethyl

RNA Ribonucleic acid

s.c. subcutaneous

VX *O*-Ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate

List of Publications

Publications

Bierwisch, A., Wille, T., Thiermann, H., Worek, F., (2016). Kinetic analysis of interactions of amodiaquine with human cholinesterases and organophosphorous compounds. *Toxicology Letters*, 246, 49-56.

Bierwisch, A., Zengerle, M., Thiermann, H., Kubik, S., Worek, F., (2014). Detoxification of alkyl methylphosphonofluoridates by an oxime-substituted β-cyclodextrin–an in vitro structure-activity study. *Toxicology Letters*, 224, 209-214.

Further publications

Mertens, M., **Bierwisch, A.**, Li, T., Gütschow, M., Thiermann, H., Wille, T., Elsinghorst, P., (2015). A novel fluorogenic probe for the investigation of free thiols: Application to kinetic measurements of acetylcholinesterase activity. *Toxicology Letters*, 244, 161-166.

von der Wellen, J., **Bierwisch, A.**, Worek, F., Thiermann, H., Wille, T., (2015). Kinetics of pesticide degradation by human fresh frozen plasma (FFP) in vitro. *Toxicology Letters*, 244, 124-128.

Brandhuber, F., Zengerle, M., Porwol, L., **Bierwisch, A.**, Koller, M., Reiter, G., Worek, F., Kubik, S., (2013). Tabun scavengers based on hydroxamic acid containing cyclodextrins. *Chemical Communications (Cambridge)*, 49, 3425-3427.

Worek, F., **Bierwisch, A.**, Wille, T., Koller, M., Thiermann, H., (2012). Kinetic interactions of a homologous series of bispyridinium monooximes (HGG oximes) with native and phosphonylated human acetylcholinesterase. *Toxicology Letters*, 212, 29-32.

Poster presentations

Bierwisch, A., Wille, T., Thiermann, H., Worek, F., Interactions of anti-malarial drugs amodiaquine and chloroquine with human cholinesterases and organophosphorus compounds. 82nd Annual Congress of the German Society of Experimental and Clinical Pharmacology and Toxicology (DGPT), Berlin, 2016.

Bierwisch, A., Leidner, A., Koller, M., Worek, F., Kubik, S., NMR spectroscopic studies of phosphonothioate binding to a resorcinarene-based deep cavitand. 15th Medical Chemical Defence Conference, München, 2015.

1 Introduction

1.1 Organophosphorus compounds

Phosphorus in its inorganic form as phosphate is mostly non-toxic and essential for all living organisms as scaffold of RNA and DNA, cellular energy balance adenosine triphosphate, component of cell membranes in form of phospholipids, and regulating cellular processes by activation or inactivation *via* phosphorylation. Likewise, organic forms of phosphorus as phosphate esters or also referred to as organophosphates and organophosphorus compounds, have a wide field of application (e. g. flame resistant oils, flame retardants, plasticizers, extreme pressure additives, and pesticides). However, organophosphates can be highly toxic to organisms, which is an advantage in pest control as pesticides, but also causes hundred thousands of accidental or voluntary poisoning and human fatalities every year [32, 38]. The advancement of more potent pesticides led to the discovery of organophosphorus nerve agents (OP), a subclass of chemical warfare agents, by Gerhard Schrader in the mid-1930s [75]. Nerve agents are divided into two main groups: G- and V-type nerve agents. Properties of G-type nerve agents ("G" is the abbreviation for Germany) include higher vapor pressure, lower hydrolytic stability, and persistency than V-type nerve agents (for "V" various meanings are possible, like venomous, vicious, and victorious). Cyclosarin (GF), sarin (GB), soman (GD), and tabun (GA) are typical representatives of G-agents (Fig. 1.1). VX is the best-known V-type nerve agent and was discovered by British and Swedish scientists in the mid-1950s [78, 80].

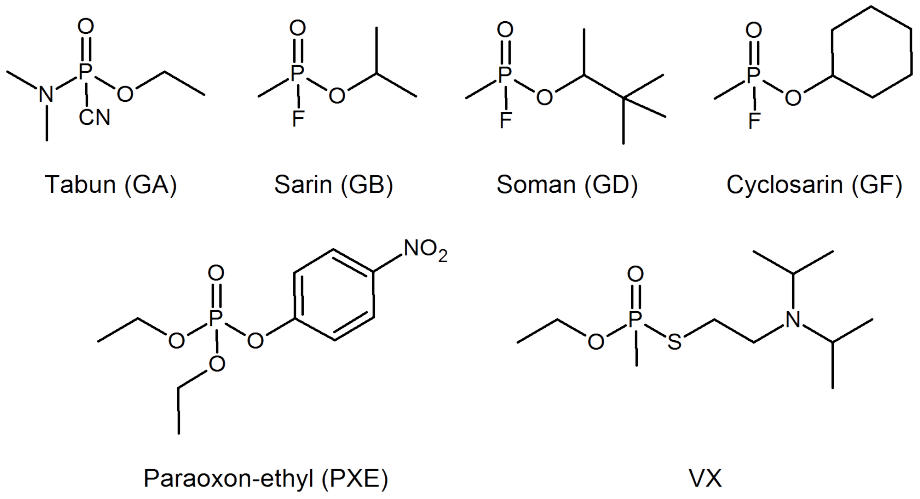

Figure 1.1: Structures of organophosphorus nerve agents and a pesticide.

The use of OP in international armed conflicts was interdicted by the Hague Conventions (1899 and 1907) and Geneva Protocol (1925) with enhancement by the Chemical Weapons Convention (1993), which additionally prohibits the production and stockpiling of chemical weapons [68]. Despite these treaties, OP still constitute

a threat to humans, e.g. deployment of tabun and sarin at the Kurdish city Halabja in the Iraq-Iran War, unintended exposure to cyclosarin and sarin of allied troops while dismantling Khamisiyah ammunition storage facility after the Gulf War, usage of sarin in terrorist attacks and VX in an assassination by the Aum Shinrikyo sect in the mid-1990s in Japan, and lately deployment of sarin in Syria affirm importance of effective medical countermeasures [12, 26, 53, 62, 65, 85].

1.2 Mode of action of organophosphorus compounds

OP' acute toxicity is based on irreversible inhibition of acetylcholinesterase (AChE, EC 1.1.1.7) by phosphylation of a serine hydroxyl group at its active site resulting in an inactive enzyme [1, 54, 81]. Consequential a surplus of neurotransmitter acetylcholine at synaptic clefts of peripheral and central nervous system with overstimulation of muscarinic and nicotinic acetylcholine receptors is triggering a cholinergic crisis, which eventually causes respiratory arrest and finally death [31, 37, 51, 66, 98]. Anticholinesterase activity of OP depends on electrophilicity of the phosphorus, which is increased by greater electron-withdrawing capacity of the nerve agent's substituent and supports the nucleophilic attack of catalytic serine which leads to phosphylated AChE [27, 29]. The stability of phosphyl-AChE-complex is more pronounced than the physiological serine-acetate intermediate and reflected in a half-life of hours to days [39]. Spontaneous hydrolysis is incapable of restoring AChE's complete functionality whereas stronger nucleophiles, termed reactivators or oximes, can remove the phosphyl moiety *via* nucleophilic attack and reactivate the enzyme (Fig. 1.2) [36, 89]. Efficacy of reactivation is influenced by the enzyme's species, structure of phosphyl moiety, structure of oxime, and a post-inhibitory process of phosphyl-AChE-complex [1, 52]. Dealkylation of an alkoxy group of phosphylated AChE through the release of an alcohol is called "aging", which is resistant to oxime reactivation [1, 5, 7, 9, 36, 61]. Aging of soman can occur in a few minutes, tabun in hours, and pesticides such as paraoxon take up to days, therefore a timely provision of medical countermeasurements is decisive [1, 79].

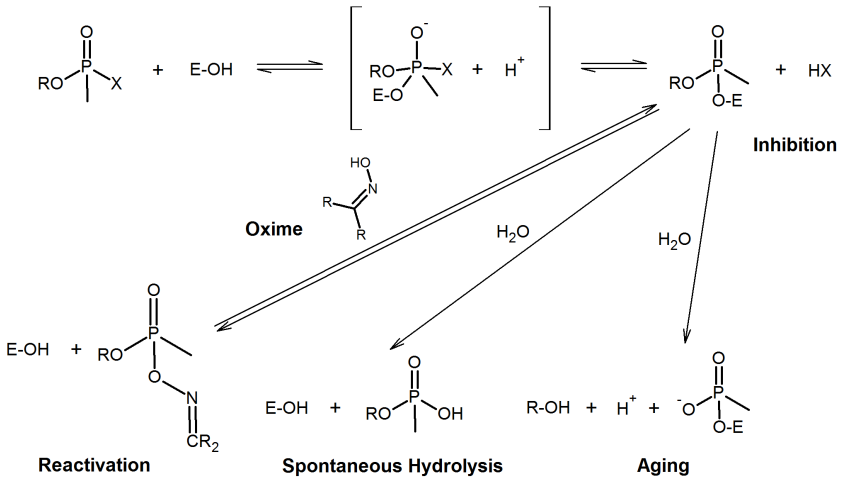

Figure 1.2: Processes of phosphorylation, aging, and reactivation between an organophosphorus compound (X stands for the leaving group) and a cholinesterase (E).

Nerve agents are present in form of a racemic mixture; therefore at least two different stereoisomers exist. The enantiomers of nerve agents have significant discrepancies in their toxicity, stability, and biological activity. (-)P-enantiomers of soman, sarin, cyclosarin, tabun, and VX exhibit a higher inhibitory potency with AChE compared to their corresponding (+)P-enantiomer [8, 21, 71, 82].

1.3 Therapy

Treatment of OP-poisoned patients currently consists of a concomitant administration of an oxime such as obidoxime, pralidoxime (2-PAM) or HI-6, and an antimuscarinic drug, e.g. atropine, often supplementd by an anticonvulsant [17, 28, 87]. The peripheral muscarinic symptoms, like sweating, salivation, miosis, bradycardia, and bronchorrhea are antagonized by atropine [33, 76]. Apart of activating muscarinic acetylcholine receptors, cholinergic crisis also triggers nicotinic acetylcholine receptors causing muscle fasciculation, muscle dysfunction, and respiratory depression [39]. Reactivation of OP-inhibited AChE *via* dephosphorylation by oximes can indirectly assist in restoring the cholinergic system [36]. However, the inevitably formed phosphylated oximes are highly reactive and may re-inhibit reactivated AChE [2, 46]. Efficacy of oximes is time-sensitive and inoperative in case of aged AChE. Hence, rapid oxime administration is crucial, otherwise solely long-lasting *de novo* synthesis can regain recovery of AChE. Clinically established oximes do not cover the broad range of OP and hardly penetrate the blood-brain barrier [20, 28, 50, 55, 95, 96]. The current treatment ensures survival, but prevention of behavioral incapacitation or neurocognitive deficits is insufficient [59]. Despite 60 years of research and synthesis of thousands of reactivators, no satisfying oxime was discovered emphasizing the need for alternative approaches and more efficient countermeasures.

1.4 Alternative approaches

1.4.1 Bioscavenger

Systemic detoxification of OP by bioscavengers before target tissues are affected is a more auspicious approach [18, 24, 74]. Apart from AChE, B-esterases such as butyrylcholinesterase (BChE, EC 3.1.1.8), carboxylesterases (EC 3.1.1.1), and neuropathy target esterase (EC 3.1.1.5) are inhibited irreversibly by OP. Consequently, by binding OP in an 1:1 molar ratio B-esterases serve as stoichiometric scavengers, thus reducing the toxic body load [15, 48, 64, 90]. Human BChE is the best developed scavenger and showed efficacy in numerous studies [18, 63, 73]. Nevertheless, detoxification of 2 x LD_{50} soman would require 200 mg of BChE, being challenging to produce, finance, and administer without interfering in metabolic and immunologic processes in the body [3, 57]. The combination of a stoichiometric bioscavenger (e.g. AChE or BChE) and an oxime, constantly reactivating the phosphorylated scavenger, is termed “pseudo-catalytic” bioscavenger [4, 44, 45, 70]. Recently, the new designed slow aging AChE mutant Y337A/F338A and oxime RS-170B showed promising performance [43]. Catalytic bioscavengers based on OP-hydrolyzing enzymes could overcome previously mentioned limitations. A single molecule of a highly active catalytic bioscavenger can detoxify theoretically indefinite OP molecules. Existing wild-type enzymes, like human paraoxonase (PON1, EC 3.1.8.1), phosphotriesterase (PTE, EC 3.1.8.1), and diisopropyl fluorophosphatase (DFPase, EC 3.1.8.2) perform with a low catalytic efficacy towards OP and prefer hydrolysis of the less toxic (+)P-enantiomers [60, 64, 84, 99]. Theoretical calculations indicate that a minimal

catalytic efficacy (k_{cat}/K_M of $>10^7$ M^{-1} min^{-1}) is required to sufficiently detoxify OP at compatible enzyme doses [3, 72]. With a prudential approach and protein engineering, PON1 mutants and PTE mutants with adequate catalytic efficacy and stereoselectivity for toxic OP enantiomers were produced [19, 30, 42]. *In vivo* studies with PON1 mutant IIG1 against 2 x LD_{50} cyclosarin s.c. and PTE mutant C23 against 2 x LD_{50} VX s.c. exhibited clearly the potential of catalytic bioscavengers [91, 92]. However, as evaluated by Worek *et al.* (2016) the bioscavengers' plasma concentration was insufficient in preventing passage of OP into tissues resulting in inhibited brain AChE activity [97]. As remedy, a higher catalytic efficacy (>5 x 10^7 M^{-1} min^{-1}) of scavengers is pursued in consideration of minimal extracorporeal load and reasonable detoxification to exposure of multiple OP LD_{50} [97]. Still, research needs to continue to descry an ideal catalytic bioscavenger, which is highly efficient, detoxifies a broad spectrum of OP, producible in large quantities, stabile at long storage, immunologically compatible to humans, and has a prolonged circulation time [56, 57, 83].

1.4.2 Small molecule scavenger

Insufficiency of standard OP therapy and major limitations of bioscavengers promoted the investigation of alternative approaches such as small molecule scavengers. Synthetic compounds are considered regarding their potential to detoxify OP *via* binding or hydrolysis. As a ring-shaped macrocycle consisting of six (α-), seven (β-) or eight (γ-) α-1,4-glycopyranoside units, cyclodextrins (CD) are due to their capability of binding organic substances in host-guest interactions favorable candidates (Fig. 1.3) [25, 35]. Characterization of CD include low toxicity, wide application in many fields, and clinical use [34, 40, 49, 69, 77]. Degradation of sarin, soman, and tabun by native CD was observed by multiple studies [22, 23, 35]. Stereospecific degradation was noted being in accordance with previous studies [22, 86].

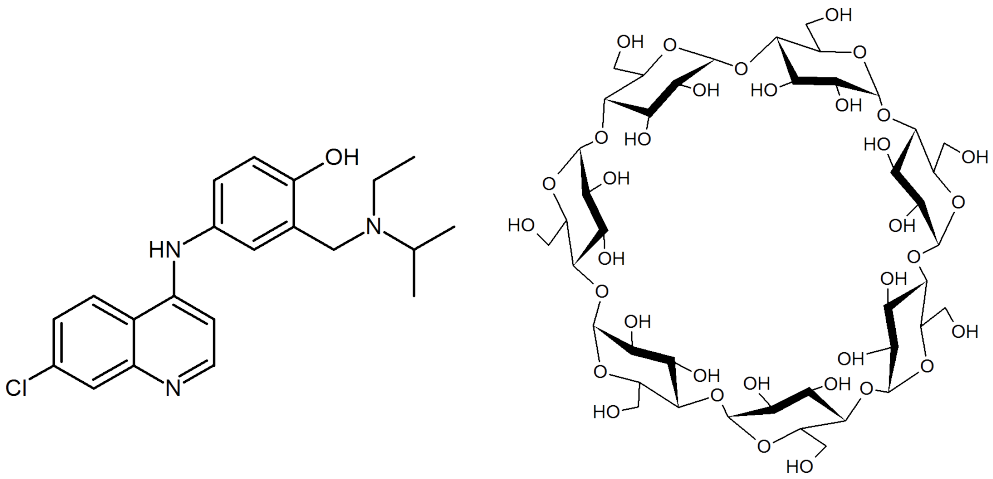

Figure 1.3: Structures of small molecules amodiaquine and β-cyclodextrin.

Superior efficacy was achieved by addition of oxime or iodosobenzoate substituents to CD cavity [47, 58, 88, 100]. A CD derivative with a pyridinium oximate in 6-position

detoxified cyclosarin rapidly *in vitro* [100]. The arrangement of a hydroxamic acid instead of an oxime at the cavity resulted in improved tabun decomposition, whereby no influence of the CD ring and no stereospecificity existed [13]. Covalent modification of CD with OP revealed a stoichiometric mode of action [14, 22]. Nevertheless, prophylactic administration of a functionalized CD against cyclosarin ensured survival and prevented systemic toxicity in anesthetized guinea pigs, confirming that OP detoxification of small molecule scavengers is promising [93]. Unfortunately, so far identification of a small molecule scavenger for broad spectrum OP degradation failed.

1.4.3 Non-oxime reactivators

Instead of acting as a scavenger, small molecules can also serve as reactivators. Due to the positive charge of the pyridinium moiety in clinically used oximes passage through the blood-brain barrier and reactivation of OP-inhibited brain AChE is hardly possible. Although a few blood-brain barrier penetrable oximes were discovered, none showed sufficient efficacy [6, 67, 94]. As a novel concept, Bhattacharjee *et al.* (2012) searched for non-oxime reactivators by virtually screening databases with an *in silico* pharmacophore modeling [10]. Amongst twelve potent non-oxime reactivators, a benzenesulfonamide derivate was as efficient as 2-PAM in preventing central nervous system damage *in vivo* in DFP-inhibited guinea pigs [11]. Additional *in silico* screenings revealed that the antimalarial drug amodiaquine might be an auspicious candidate because it can reactivate PXE-inhibited AChE (Fig. 1.3) [41]. In further investigations, mannich phenols in particular 4-amino-2-(diethylaminomethyl)phenol (ADOC) were accounted for reactivation of PXE-inhibited AChE. Recently, ADOC and related compounds were tested regarding their reactivation potential *in vitro* and *in vivo*, but failed expectations [16]. Unfortunately, additional investigations regarding amodiaquine's potential were neglected.

1.5 Objectives of the presented thesis

Despite intensified research for decades, major drawbacks in the standard treatment of OP poisoning could not be overcome, hence novel therapeutic approaches based on bioscavengers or small molecule scavengers are currently closely investigated. The presented thesis focuses on small molecules as scavengers or non-oxime reactivators and their potential as therapeutics in OP poisoning.

For developing a scavenger with the potential to detoxify a broad spectrum of OP more rapidly, data about preferred conditions for complex formation and desirable structure of guests is needed. Therefore, a structure-activity study with a functionalized CD and various alkoxy residues of methylphosphonofluoridates (sarin derivatives) was conducted, specified in chapter 2.1 (first publication).

Additionally, another small molecule, the antimalarial drug amodiaquine, was investigated for its ability to detoxify OP, but failed expectations. However, reactivation of OP-inhibited cholinesterases by amodiaquine was observed. Thus, amodiaquine was further characterized by focusing on interactions between amodiaquine and human cholinesterases (AChE and BChE) with different OP and relating data towards reactivation potential, efficacy, and treatment options, which give a new insight in OP therapy and is dealt with in chapter 2.2 (second publication).

2 Publications

2.1 Detoxification of alkyl methylphosphonofluoridates by an oxime-substituted β-cyclodextrin - An in vitro structure-activity study

A. Bierwisch, M. Zengerle, H. Thiermann, S. Kubik, F. Worek, *Toxicology Letters* **2014**, 224, 209-214.

The concomitant treatment of OP poisoning with atropine and an oxime has many limitations. Numerous attempts were undertaken to prevent OP from exerting their acute toxicity and neuronal malfunction without a breakthrough. Besides bioscavengers, small molecule scavengers are most widely studied towards their ability to detoxify OP. Within a collaboration between the TU Kaiserslautern and the Bundeswehr Institute of Pharmacology and Toxicology a diversity of cyclodextrins were synthesized in Kaiserslautern and tested for their efficacy to bind or degrade OP in München. A small molecule scavenger in form of a β-cyclodextrin bearing a nucleophilic oximate group in 6-position showed high efficiency in cyclosarin detoxification *in vitro* and *in vivo* [93, 100].
Nevertheless, a small molecule scavenger detoxifying a broad range of OP is preferable. To develop the desired scavenger, detailed information about formation of inclusion complexes between β-cyclodextrin and OP is essential. Therefore the 6-pyridinium oximate substituted β-cyclodextrin was extensively tested *in vitro* with a broad range of methylphosphonofluoridates (sarin derivatives) varying in alkoxy residue to determine structure-activity relationships. By applying a modified Ellman assay, detoxification kinetics were determined spectrophotometrically. Degradation of alkyl methylphosphonofluoridates was significantly better with the oxime substituted β-cyclodextrin than native β-cyclodextrin, thereby conforming the essential role of the nucleophilic group by OP degradation. An increased detoxification rate by the substituted β-cyclodextrin was achieved with bulkier alkyl methylphosphonofluoridates (publication 2.1, Table 2) *iso*-pentylsarin (k_{detox} = 118.5 ± 8.6 x 10^{-2} M^{-1} min^{-1}), except for soman (k_{detox} = 1.0 ± 0.1 x 10^{-2} M^{-1} min^{-1}). This led to the thesis that a faster OP degradation is accompanied by a more stable complex formation between substituted β-cyclodextrin and sarin derivatives. Data from a previous study, in which stability constants of complex formation between β-cyclodextrin and alcohol residue of OP were correlated, supported this hypothesis (publication 2.1, Table 2). Despite the similar structure to sarin, the nerve agent soman was degraded significantly slower. Steric hindrance and increased existence of more toxic and stabile (-)P-enantiomers of soman could be the reason for low detoxification of this phosphonate by the oxime substituted β-cyclodextrin.

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Detoxification of alkyl methylphosphonofluoridates by an oxime-substituted β-cyclodextrin – An in vitro structure–activity study

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HIGHLIGHTS

- The ability of a β-cyclodextrin derivative to detoxify 11 sarin derivatives was assessed.
- Detoxification is always faster than hydrolysis of the respective organophosphonate.
- A structure–activity relationship was established.
- Rates were correlated with stabilities of the respective cyclodextrin complexes.

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In vitro study

ABSTRACT

Detoxification rates of a series of alkyl methylphosphonofluoridates by an oxime-substituted β-cyclodextrin (β-CD) were assessed quantitatively by using an AChE inhibition assay. The cyclodextrin (CD) derivative was identified in previous work as a highly active cyclosarin scavenger. Here, a structure–activity relationship was established by investigating the effect of this CD on the detoxification of sarin derivatives differing in the structure of the alkoxy residue. The results show that detoxification rates correlate with the steric bulk and chain length of the alkoxy group in the organophosphonate (OP). OPs with larger, more bulky residues are detoxified more rapidly, with the exception of soman, which is bearing a pinacolyloxy side chain. In addition, the substituted CD was in every case more active than unsubstituted, native β-CD with up to a 400-fold difference. Comparing the kinetic results obtained with the known thermodynamic stabilities of related β-CD complexes indicate that detoxification rates generally increase when the alkoxy residue on the OP is exchanged by a residue, which forms a more stable complex with β-CD. This correlation lends support to the proposed mode of action of the substituted CD, involving initial complexation of the OP followed by reaction between the CD and the OP. The moderate to high efficacy on the detoxification of sarin derivatives suggests the potential applicability of this CD as a small molecule scavenger for G-type nerve agents.

1. Introduction

Every year 2–5 million people suffer from poisonings by pesticides (accidental or suicidal) and up to 220,000 fatal cases are estimated (Karalliedde and Szinicz, 2001). Pesticides are a heterogeneous group of synthetic chemicals that include organophosphorous compounds (OP) whose toxicity is due to the covalent modification and the resulting inhibition of the enzyme acetylcholinesterase (AChE) (Taylor et al., 1995). AChE inhibition causes accumulation of the neurotransmitter acetylcholine leading to disturbance of numerous body functions and eventually to death due to respiratory arrest. Standard treatment of OP poisoning includes an anti-muscarinic compound, e.g. atropine, and an oxime as AChE reactivator (Wiener and Hoffman, 2004). However, numerous limitations are associated with standard treatment protocols. Antimuscarinic drugs do not counteract OP effects at nicotinic receptors, for example. Moreover, oximes hardly cross the blood–brain-barrier (Eyer and Worek, 2007; Aurbek et al., 2009) and are generally insufficient in reactivating AChE inhibited by certain nerve agents, e.g. soman and tabun (Eyer and Worek, 2007; Worek et al., 2004). Also reactivation by oximes of inhibited AChE containing an aged OP residue resulting from hydrolysis of an alkoxy residue on the phosphorus atom is not feasible (Aldridge and Reiner, 1972; Worek et al., 1996). Finally, treatment of

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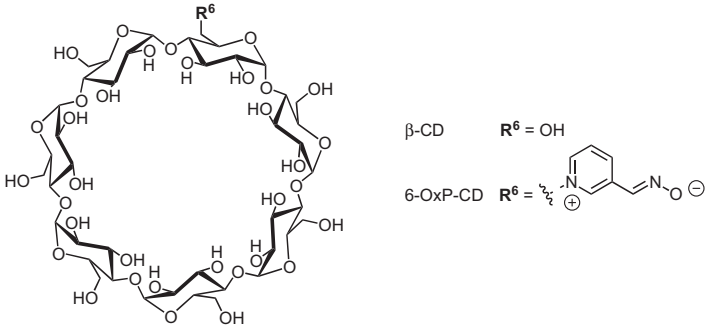

Fig. 1. Chemical structures of β-CD and 6-OxP-CD.

poisonings with persistent nerve agents such as VX that are only slowly cleared from the organism is particularly difficult (Reiter et al., 2008, 2011; Tenberken et al., 2010). In order to overcome these limitations, new therapeutic approaches are urgently needed. One research effort is directed at the development of stoichiometric or catalytic enzymes that can act as scavengers by binding or degrading OPs *in vivo* prior to the appearance of any toxic effects (Masson and Rochu, 2009). Synthetic compounds, so-called small molecule scavengers, can serve similar purposes.

Promising lead structures for the development of small molecule scavengers are cyclodextrins (CDs; Fig. 1), cyclic oligosaccharides containing glycopyranose subunits linked by α(1–4) glycosidic linkages. Negligible toxicity and the relative ease with which CDs can be structurally modified led to application of this class of compounds in many different areas (Szejtli, 1998). A characteristic feature of CDs is their ability to include small hydrophobic compounds in the apolar interior of the macrocyclic cavity. Stability of the complexes formed depends on ring size of the CD and on the structure of the substrate. In general, substrates that efficiently occupy the available space of the CD cavity are bound best. In the case of the seven-membered β-cyclodextrin (β-CD), for example, adamantane derivatives are very good substrates (Rekharsky and Inoue, 1998). Complex stability is determined by a combination of intermolecular interactions of which the most important are vander-Waals interactions (Bergeron et al., 1978), hydrogen bonding (Tabushi et al., 1978), dipole–dipole interactions (Gelb et al., 1981; Bergeron et al., 1977), and the hydrophobic effect (Connors, 1997).

Several studies have shown that native CDs can also degrade the nerve agents soman and sarin (Hennrich and Cramer, 1965; Van Hooidonk and Groos, 1970; Desiré and Saint-André, 1987). Degradation efficiency could be improved by arranging functional groups around the CD cavity that actively participate in the reaction. Examples include functionalization of β-CD with 2-iodosobenzoates or oximes, which afforded scavengers for cyclosarin, tabun, or paraoxon (Masurier et al., 2005; Wille et al., 2009; Le Provost et al., 2011; Zengerle et al., 2011). Zengerle et al. (2011) showed that β-CD derivative 6-OxP-CD (Fig. 1) with a pyridinium oximate in 6-position of one glucose unit detoxifies the toxic (−)-enantiomer of cyclosarin within seconds and the less toxic (+)-enantiomer with a half-time of 43 s under appropriate conditions. However, the same CD had only a small effect on tabun detoxification (Brandhuber et al., 2012).

In order to investigate whether detoxification activity of 6-OxP-CD is limited to cyclosarin or if this compound is able to detoxify other alkyl methylphosphonofluoridates with similar efficiency, rates of 6-OxP-CD-mediated detoxification of a series of sarin derivatives differing in the alcohol component (Fig. 2) were assessed by using an enzymatic *in vitro* assay. These measurements allowed establishing a structure–activity relationship demonstrating that sarin derivatives with larger, bulky alkyl substituents that presumably interact with 6-OxP-CD more strongly are detoxified more rapidly than OPs with small alkyl residues.

2. Materials and methods

2.1. Materials

Acetylthiocholine iodide (ATCh), β-cyclodextrin, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and tris(hydroxymethyl)aminomethane (TRIS) were obtained from Sigma–Aldrich (Taufkirchen, Germany). Disodium hydrogenphosphate dihydrate, potassium dihydrogenphosphate, and hydrochloric acid were purchased from Roth (Karlsruhe, Germany). Sarin, cyclosarin, soman, ethylsarin, *n*-propylsarin, *iso*-butylsarin, *n*-butylsarin, *iso*-pentylsarin, *n*-pentylsarin, *neo*-pentylsarin and *sec*-pentylsarin (Fig. 2; >95% by GC–MS, ^{1}H NMR and ^{31}P NMR) were made available by the German Ministry of Defence (Bonn, Germany). 6-OxP-CD was synthesized according to Zengerle et al. (2011). All other chemicals were purchased from Merck Eurolab (Darmstadt, Germany).

Stock solutions of sarin, soman, cyclosarin (0.1%, v/v), and sarin analogs (1%, v/v) were prepared in acetonitrile, stored at room temperature, and diluted appropriately with Tris–HCl buffer (0.1 M, pH 7.4) just before use. CD stock solutions (10 mM) were prepared daily in Tris–HCl buffer and diluted as required with Tris–HCl buffer prior to use.

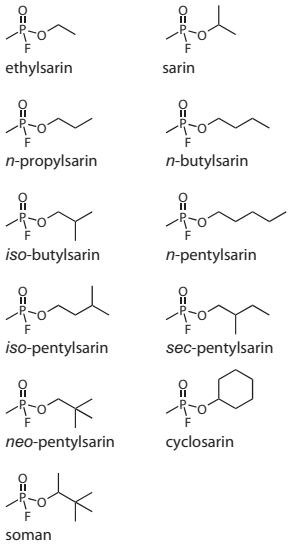

Fig. 2. Chemical structures of the alkyl methylphosphonofluoridates tested.

Hemoglobin-free human erythrocyte ghosts served as AChE source and were prepared according to Dodge et al. (1963) with minor modifications (Worek et al., 2002). Briefly, heparinized human whole blood was centrifuged (3000 × *g*, 10 min) for plasma removal. The erythrocytes were washed three times with two volumes of phosphate buffer (0.1 M, pH 7.4). To facilitate hemolysis, the packed erythrocytes were diluted in 20 volumes of hypotonic phosphate buffer (6.7 mM, pH 7.4) and centrifuged at 50,000 × *g* (30 min, 4 °C). After two additional washing cycles the pellet was re-suspended in phosphate buffer (0.1 M, pH 7.4) and the initial AChE activity was adjusted. Aliquots of the erythrocyte ghosts were stored at −80 °C. Prior to use, thawed ghosts were homogenized on ice with Sonoplus HD 2070 ultrasonic homogenator (Bandelin electronic, Berlin, Germany) twice for 5 s with a 20 s interval to achieve a homogenous matrix for kinetic studies.

2.2. AChE assay

AChE activities were determined spectrophotometrically (UV mc 2, SAFAS, Monaco) with a modified Ellman assay at 412 nm (Ellman et al., 1961; Eyer et al., 2003; Worek et al., 1999). Polystyrol cuvettes (3.18 mL) contained ATCh (50 μL, 0.45 mM) as substrate and DTNB (100 μL, 0.3 mM) as a chromogen in 0.1 M Tris–HCl buffer (3.0 mL). All experiments were performed at 37 °C and the given concentrations refer to final concentrations.

2.3. Determination of detoxification constants

Detoxification kinetics of the different OPs by β-CD or 6-OxP-CD were investigated by an AChE inhibition assay (Wille et al., 2013). 50 μL β-CD or 6-OxP-CD (0.1 M Tris–HCl buffer, pH 7.4) were mixed with 10 μL OP (0.1 M Tris–HCl buffer) and 140 μL Tris–HCl (0.1 M, pH 7.4; $t = 0$). The resulting CD concentrations were 2.5 mM, except for cyclosarin and pentylsarin analogs (0.5 mM) and the final OP concentrations were 50 mM (sarin, soman, cyclosarin), 30 mM (ethylsarin), 6 mM (*n*-propylsarin, *n*-butylsarin) and 5 mM for the remaining OPs. After specified time intervals, 20 μL aliquots were transferred to 37 °C thermostated cuvettes for recording the inhibition of AChE activity. Corresponding experiments with OPs in Tris–HCl buffer were performed to determine the spontaneous hydrolysis kinetics.

The recorded inhibition curves for each sample were analyzed by non-linear regression analysis to calculate first-order inhibition rate constants k_{obs} (Fig. 3A; Aurbek et al., 2006). These values were plotted *vs.* the time of incubation to which they correspond. First-order detoxification constants k_1 were then obtained by fitting the resulting decay curves (Fig. 3B and C) and correcting for spontaneous hydrolysis of the corresponding OP. Finally, pseudo second-order detoxification constants k_{detox} were calculated on the basis of Eq. (1).

$$k_{detox} = \frac{k_1}{c(CD)} \quad (1)$$

2.4. Data analysis

Kinetic constants were calculated from the experimental data by non-linear regression analysis using Prism 5.04 (GraphPad Software, San Diego, CA, USA). All experiments were performed in duplicate.

3. Results

Incubation of β-CD or 6-OxP-CD with all sarin derivatives considered in this study resulted in a time-dependent mono-exponential decrease of the inhibition potency of the solutions toward human AChE (Fig. 3 and Table 1). From these data, first-order detoxification constants could be calculated. These detoxification rate constants were corrected by the rates of spontaneous hydrolysis of the respective OP, which ranged between 0.010 min^{-1} (GB, GD, GF) and 0.034 min^{-1} (ethylsarin), to yield k_1. The k_1 values thus reflect the extent to which detoxification rate is faster than spontaneous hydrolysis of the respective OP. Due to differences in the rates of detoxification in the series of the OPs studied, different 6-OxP-CD and β-CD concentrations had to be used for the measurements. To allow comparison of the data, pseudo second-order detoxification constants k_{detox} were therefore calculated from the ratios of k_1 and the corresponding CD concentrations (Table 2).

Tables 1 and 2 show that the structure of the alcohol residue on the OP affects detoxification rate in a characteristic manner. In addition, the substituted CD derivative 6-OxP-CD is a significant better scavenger than native β-CD, independent of OP structure. The oxime residue on the CD accelerates OP detoxification over the rate observed in the presence of β-CD between a factor of 4.1 (for soman) and 98.8 (for cyclosarin). Table 2 shows that 6-OxP-CD most effectively detoxifies *iso*-pentylsarin and cyclosarin, while the effect is approximately 100-fold lower in the case of soman and sarin. A comparable pattern was observed for the β-CD mediated detoxification of these OPs although differences are markedly lower.

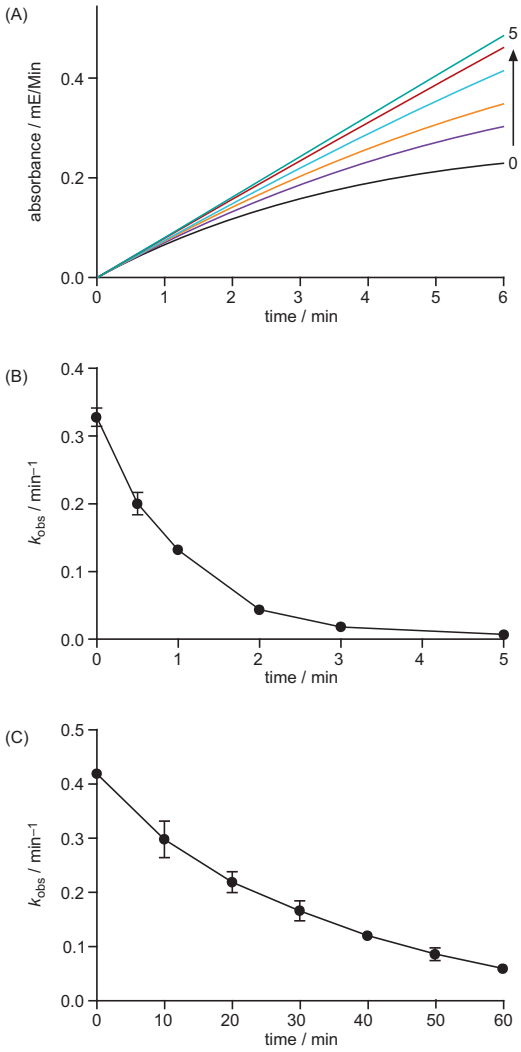

Fig. 3. Time-dependent detoxification mediated by β-CD and 6-OxP-CD of *n*-propylsarin as an example. (A) Aliquots, taken from an incubate of *n*-propylsarin and 6-OxP-CD after 0–5 min, were transferred to a cuvette for measurement of the inhibition kinetics with human AChE by an Ellman assay followed by non-linear regression analysis for the determination of k_{obs}; plots of k_{obs} *vs.* the time at which k_1 was determined to calculate the first-order rate constant for the detoxification of *n*-propylsarin by 6-OxP-CD (B) and β-CD (C). Data shown in (B) and (C) are means of two replicates ± the deviation of the two measurements. Note the different time scales in (B) and (C).

Table 1
First-order rate constants k_1 describing the detoxification rates corrected by the rates of spontaneous OP hydrolysis of the detoxification of different sarin derivatives mediated by 6-OxP-CD and β-CD.

OP	6-OxP-CD k_1 (min^{-1})	β-CD k_1 (min^{-1})	CD concentration (mM)
Ethylsarin	0.46 ± 0.04	0.002 ± 0.004	2.5
n-Propylsarin	0.96 ± 0.02	0.002 ± 0.002	2.5
Sarin	0.28 ± 0.02	0.004 ± 0.001	2.5
n-Butylsarin	3.24 ± 0.37	0.044 ± 0.007	2.5
iso-Butylsarin	2.51 ± 0.06	0.039 ± 0.003	2.5
n-Pentylsarin	1.98 ± 0.24	0.031 ± 0.005	0.5
iso-Pentylsarin	5.92 ± 0.43	0.080 ± 0.001	0.5
sec-Pentylsarin	4.94 ± 0.28	0.041 ± 0.002	0.5
neo-Pentylsarin	4.32 ± 0.25	0.074 ± 0.002	0.5
Soman	0.25 ± 0.01	0.059 ± 0.004	2.5
Cyclosarin	5.56 ± 0.72	0.056 ± 0.001	0.5

All constants were determined by non-linear regression analysis of k_{obs} *vs.* time. Data were obtained from duplicate runs and are given as means ± SD. Note that different CD concentrations were used in the experiments with pentylsarin analogs and cyclosarin.

4. Discussion

The results of the present study indicate that the β-CD derivative 6-OxP-CD has the ability to detoxify a series of different alkyl methylphosphonofluoridates albeit at markedly different rates. This CD derivative therefore meets an important prerequisite for the use of small molecule or enzyme-based scavengers, namely to act against a variety of relevant and structurally different OPs (Masson and Rochu, 2009).

In the case of CD-based scavengers it is generally assumed that detoxification involves initial incorporation of part of the OP into the CD cavity, which brings a hydroxyl group or a nucleophilic substituent on the ring in close proximity to the OP phosphorus atom (Van Hooidonk and Breebaart-Hansen, 1970; Desiré and Saint-André, 1986; Desiré and Saint-André, 1987; Hoskin et al., 1999; Wille et al., 2009; Le Provost et al., 2011; Müller et al., 2011; Zengerle et al., 2011). Subsequent reaction between the CD and the OP then leads to deactivation. For a given CD derivative such as 6-OxP-CD detoxification rates should therefore depend on the stability of the complex formed between the CD and an OP as well as on structural parameters of this complex. Thermodynamic stability determines the amount of complex in solution: the higher stability, the more complex is present and the faster the reaction between CD and OP. However, if the nucleophilic group on the CD and the OP phosphorous atom are arranged in the complex formed between the two components in a fashion that does not easily allow reaction, detoxification rates are expected to be low even if a stable complex is formed. In certain cases, CDs can even protect OPs from being detoxified because the OP is protected in the CD complex from reacting with water (Cruickshank et al., 2013).

Unfortunately, no information is available about the stabilities of complexes between β-CD and sarin derivatives. However, stabilities have been determined for complexes between the alcohol components of the sarin derivatives investigated in this work and β-CD (Rekharsky and Inoue, 1998). Since these sarin derivatives differ only in the alcohol part, the corresponding alcohols could be regarded as suitable analogs. A particularly useful study in this respect is the one of the Matsui and Mochida (1979), in which interactions between β-CD and the whole set of alcohols underlying the sarin derivatives investigated here were quantified under comparable conditions. The stability constants reported in the work of Matsui and Mochida are included in Table 2.

Table 2 shows that, indeed, sarin derivatives are generally detoxified more rapidly if their alcohol component forms a more stable complex with the β-CD ring. This trend is independent of whether the β-CD is substituted or not. Matsui und Mochida assumed that 1-pentanol could fit completely into the β-CD cavity, which agrees well with the fact that pentylsarin derivatives are detoxified most rapidly, with the highest rates of detoxification observed for *iso*-pentylsarin. *iso*-Pentylsarin is detoxified even slightly faster than what was previously observed for cyclosarin (Zengerle et al., 2011).

While this trend supports the assumption that stability of the complexes formed between these sarin derivatives and β-CD affects detoxification rate there are also deviations. Most importantly, of all the sarin derivatives investigated soman is detoxified with the lowest rate although the pinacolyl residue is expected to efficiently interact with β-CD. *neo*-Pentylsarin, which lacks the methyl group at the carbon atom bound to the alcohol oxygen with respect to soman, is detoxified considerably faster. In the case of soman it is likely that structural parameters of the complex (e.g. incomplete inclusion of the alcohol residue into the CD cavity caused by steric effects of the methyl group next to the oxygen atom) are responsible for inhibition of the reaction between the phosphonate and a nucleophilic group on the ring. In addition, one has to consider that soman exists as a mixture of four stereoisomers of which the two P(−) enantiomers have a higher inhibitory potency toward AChE than the P(+) enantiomers (De Jong et al., 1988). It cannot be ruled out that 6-OxP-CD mediated

Table 2
Second-order detoxification constants k_{detox} of the detoxification of different sarin derivatives mediated by 6-OxP-CD and β-CD.

OP	6-OxP-CD $k_{detox} \times 10^{-2}$ ($M^{-1}\ min^{-1}$)	β-CD $k_{detox} \times 10^{-2}$ ($M^{-1}\ min^{-1}$)	k_{detox} (6OxP-CD)/k_{detox} (β-CD)	log K_a[a]
Ethylsarin	1.8 ± 0.1	0.009 ± 0.015	211	–0.03
n-Propylsarin	3.8 ± 0.1	0.009 ± 0.005	418	0.57
Sarin	1.1 ± 0.1	0.017 ± 0.001	65	0.58
n-Butylsarin	13.0 ± 1.5	0.175 ± 0.029	74	1.22
iso-Butylsarin	10.0 ± 2.5	0.157 ± 0.011	64	1.62
n-Pentylsarin	39.7 ± 4.9	0.622 ± 0.100	64	1.80
iso-Pentylsarin	118.5 ± 8.6	1.591 ± 0.009	74	2.25
sec-Pentylsarin	98.9 ± 5.5	0.825 ± 0.044	120	2.08
neo-Pentylsarin	86.5 ± 5.0	1.483 ± 0.034	58	2.76
Soman	1.0 ± 0.1	0.237 ± 0.015	4	2.75
Cyclosarin	111.2 ± 14.4	1.125 ± 0.027	99	2.70

[a] Stability constants log K_a of the complexes between β-CD and the corresponding alcohol component of the OP. Data taken from Matsui and Mochida (1979).

detoxification of the P(−)C(−) and P(−)C(+) soman enantiomers proceeds at significantly different rates. Indeed, cyclosarin detoxification mediated by 6-OxP-CD is highly enantioselective indicating that the absolute configuration of the OP also has an influence on the rates of OP deactivation (Zengerle et al., 2011).

The fact that 6-OxP-CD exhibits a substantially higher detoxification activity for all sarin derivatives studied in comparison with native β-CD is a strong indication that the nucleophilic oxime group in 6-OxP-CD participates in the reaction. It should be noted that the effect of the oxime group is smallest in the case of soman detoxification, which again suggests that the nucleophilic group in 6-OxP-CD is not well positioned to efficiently react with this OP in contrast to other sarin derivatives.

In conclusion, this systematic study indicates that there exists a clear structure activity relationship in the 6-OxP-CD mediated deactivation of sarin derivatives. Activities could be correlated with the stability of the sarin complexes, but seem to be also influenced by structural parameters of the complexes formed. The moderate to high efficacy of 6-OxP-CD on sarin detoxification suggests the potential applicability of this compound as a small molecule scavenger for G-type nerve agents.

Conflict of interest

The concept and execution of the project, the interpretation of the results, and the preparation of the manuscript were under the control of the authors and have not been influenced by the German Ministry of Defence.

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References

Aldridge, W.N., Reiner, E., 1972. Enzyme Inhibitors as Substrates-Interactions of Esterases with Esters of Organophosphorus and Carbamic Acids. North-Holland Publishing Company, Amsterdam, London.

Aurbek, N., Thiermann, H., Szinicz, L., Eyer, P., Worek, F., 2006. Analysis of inhibition, reactivation and aging kinetics of highly toxic organophosphorus compounds with human and pig acetylcholinesterase. Toxicology 224, 91–99.

Aurbek, N., Worek, F., Thiermann, H., 2009. Aktuelle Aspekte in der Behandlung der phosphororganischen Vergiftung. Wehrmed. Mschr. 53, 340–349.

Bergeron, R.J., Channing, M.A., Gibeily, G.J., Pillor, D.M., 1977. Disposition requirements for binding in aqueous solution of polar substrates in the cyclohexaamylose cavity. J. Am. Chem. Soc. 99, 5146–5151.

Bergeron, R.J., Pillor, D.M., Gibeily, G., Roberts, W.P., 1978. Thermodynamics of cycloamylose-substrate complexation. Bioorg. Chem. 7, 263–271.

Brandhuber, F., Zengerle, M., Porwol, L., Tenberken, O., Thiermann, H., Worek, F., Kubik, S., Reiter, G., 2012. Detoxification of tabun at physiological pH mediated by substituted β-cyclodextrin and glucose derivatives containing oxime groups. Toxicology 302, 163–171.

Connors, K.A., 1997. The stability of cyclodextrin complexes in solution. Chem. Rev. 97, 1325–1358.

Cruickshank, D.L., Rougier, N.M., Vico, R.V., Bourne, S.A., Buján, E.I., Caira, M.R., de Rossi, R.H., 2013. Inclusion of the insecticide fenitrothion in dimethylated-β-cyclodextrin: unusual guest disorder in the solid state and efficient retardation of the hydrolysis rate of the complexed guest in alkaline solution. Beilstein J. Org. Chem. 9, 106–117.

De Jong, L.P.A., Van Dijk, C., Benschop, H.P., 1988. Hydrolysis of the four stereoisomers of soman catalyzed by liver homogenate and plasma from rat, guinea pig and marmoset, and human plasma. Biochem. Pharmacol. 37, 2939–2948.

Desiré, B., Saint-André, S., 1986. Interaction of soman with beta-cyclodextrin. Fundam. Appl. Toxicol. 7, 646–657.

Desiré, B., Saint-André, S., 1987. Inactivation of sarin and soman by cyclodextrins in vitro. Experientia 43, 395–397.

Dodge, J.T., Mitchell, C., Hanahan, D.J., 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Arch. Biochem. Biophys. 100, 119–130.

Ellman, G.L., Courtney, K.D., Andres Jr., V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Eyer, P., Worek, F., Kiderlen, D., Sinko, G., Stuglin, A., Simeon-Rudolf, V., Reiner, E., 2003. Molar absorption coefficients for the reduced Ellman reagent: reassessment. Anal. Biochem. 312, 224–227.

Eyer, P., Worek, F., 2007. Oximes. In: Marrs, T.C., Maynard, R.L., Sidell, F.R. (Eds.), Chemical Warfare Agents: Toxicology and Treatment. John Wiley & Sons Ltd., Chichester, pp. 305–329.

Gelb, R.I., Schwartz, L.M., Cardelino, B., Fuhrman, H.S., Johnson, R.F., Laufer, D.A., 1981. Binding mechanisms in cyclohexaamylose complexes. J. Am. Chem. Soc. 103, 1750–1757.

Hoskin, F.C.G., Steeves, D.M., Walker, J.E., 1999. Substituted cyclodextrin as a model for a quad enzyme that hydrolyzes the nerve gas soman. Biol. Bull. 197, 284–285.

Hennrich, N., Cramer, F., 1965. Inclusion compounds. 18. The catalysis of the fission of pyrophosphates by cyclodextrin. A model reaction for the mechanism of enzymes. J. Am. Chem. Soc. 87, 1121–1126.

Karalliedde, L., Szinicz, L., 2001. Management of organophosphorous compound poisoning. In: Karalliedde, L., Feldman, S., Henry, J., Marrs, C.T. (Eds.), Organophosphates and Health. Imperial College Press, London, p. 257.

Le Provost, R., Wille, T., Louise, L., Masurier, N., Müller, S., Reiter, G., Renard, P.-Y., Lafont, O., Worek, F., Estour, F., 2011. Optimized strategies to synthesize β-cyclodextrin-oxime conjugates as a new generation of organophosphate scavengers. Org. Biomol. Chem. 9, 3026–3032.

Masson, P., Rochu, D., 2009. Catalytic bioscavengers against toxic esters, an alternative approach for prophylaxis and treatments of poisonings. Acta Naturae 1, 68–78.

Masurier, N., Estour, F., Froment, M.T., Lefèvre, B., Debouzy, J.C., Brasme, B., Masson, P., Lafont, O., 2005. Synthesis of 2-substituted β-cyclodextrin derivatives with a hydrolytic activity against the organophosphorylester paraoxon. Eur. J. Med. Chem. 40, 615–623.

Matsui, Y., Mochida, K., 1979. Binding forces contributing to the association of cyclodextrin with alcohol in an aqueous solution. Bull. Chem. Soc. Jpn. 52, 2808–2814.

Müller, S., Koller, M., Le Provost, R., Lafont, O., Estour, F., Wille, T., Thiermann, H., Worek, F., Reiter, G., 2011. In vitro detoxification of cyclosarin (GF) by modified cyclodextrins. Toxicol. Lett. 200, 53–58.

Reiter, G., Mikler, J., Hill, I., Weatherby, K., Thiermann, H., Worek, F., 2008. Chromatographic resolution, characterisation and quantification of VX enantiomers in hemolysed swine blood samples. J. Chromatogr. B 873, 86–94.

Reiter, G., Mikler, J., Hill, I., Weatherby, K., Thiermann, H., Worek, F., 2011. Simultaneous quantification of VX and its toxic metabolite in blood and plasma samples and its application for in vivo and in vitro toxicological studies. J. Chromatogr. B 879, 2704–2713.

Rekharsky, M.V., Inoue, Y., 1998. Complexation thermodynamics of cyclodextrins. Chem. Rev. 98, 1875–1918.

Szejtli, J., 1998. Introduction and general overview of cyclodextrin chemistry. Chem. Rev. 98, 1743–1753.

Taylor, P., Radic, Z., Hosea, N.A., Camp, S., Marchot, P., Berman, H.A., 1995. Structural basis for the specificity of cholinesterase catalysis and inhibition. Toxicol. Lett. 82–83, 453–458.

Tabushi, I., Kiyosuke, Y., Sugimoto, T., Yamamura, K., 1978. Approach to the aspects of driving force of inclusion by α-cyclodextrin. J. Am. Chem. Soc. 100, 916–919.

Tenberken, O., Worek, F., Thiermann, H., Reiter, G., 2010. Development and validation of a sensitive gas chromatography–ammonia chemical ionization mass spectrometry method for the determination of tabun enantiomers in hemolysed blood and plasma of different species. J. Chromatogr. B 878, 1290–1296.

Van Hooidonk, C., Breebaart-Hansen, J.C.A.E., 1970. Stereospecific reaction of isopropyl methyl-phosphonofluoridate (sarin) with β-cyclodextrin: a model for enzyme inhibition. Recl. Trav. Chim. Pays-Bas 89, 289–299.

Van Hooidonk, C., Groos, C.C., 1970. Model studies for enzyme inhibition. Part II: The stereospecific reaction of isopropyl p-nitrophenyl methylphosphonate with α-cyclodextrin in aqueous alkaline media. Recl. Trav. Chim. Pays-Bas 89, 845–856.

Wiener, S.W., Hoffman, R.S., 2004. Nerve agents: a comprehensive review. J. Intensive Care Med. 19, 22–37.

Wille, T., Tenberken, O., Reiter, G., Müller, S., Le Provost, R., Lafont, O., Estour, F., Thiermann, H., Worek, F., 2009. Detoxification of nerve agents by a substituted β-cyclodextrin: application of a modified biological assay. Toxicology 265, 96–100.

Wille, T., Scott, C., Thiermann, H., Worek, F., 2013. Detoxification of G- and V-series nerve agents by the phosphotriesterase OpdA. Biocat. Biotrans. 30, 03–208.

Worek, F., Kirchner, T., Bäcker, M., Szinicz, L., 1996. Reactivation by various oximes of human erythrocyte acetylcholinesterase inhibited by different organophosphorus compounds. Arch. Toxicol. 70, 497–503.

Worek, F., Mast, U., Kiderlen, D., Diepold, C., Eyer, P., 1999. Improved determination of acetylcholinesterase activity in human whole blood. Clin. Chim. Acta 288, 73–90.

Worek, F., Reiter, G., Eyer, P., Szinicz, L., 2002. Reactivation kinetics of acetylcholinesterase from different species inhibited by highly toxic organophosphates. Arch. Toxicol. 76, 523–529.

Worek, F., Thiermann, H., Szinicz, L., Eyer, P., 2004. Kinetic analysis of interactions between human acetylcholinesterase, structurally different organophosphorus compounds and oximes. Biochem. Pharmacol. 68, 2237–2248.

Zengerle, M., Brandhuber, F., Schneider, C., Worek, F., Reiter, G., Kubik, S., 2011. Highly efficient cyclosarin degradation mediated by a β-cyclodextrin derivative containing an oxime-derived substituent. Beilstein J. Org. Chem. 7, 1543–1554.

2.2 Kinetic analysis of interactions of amodiaquine with human cholinesterases and organophosphorus compounds

A. Bierwisch, T. Wille, H. Thiermann, F. Worek, *Toxicology Letters* **2016**, 246, 49-56.

Inefficacy of oximes against several OP resulted in investigation of small molecules as potential non-oxime reactivators. While performing *in silico* screening of databases, several research groups identified new potential non-oxime reactivators. Among the candidates, amodiaquine was most auspicious and in fact reactivated PXE-inhibited AChE. Unfortunately, closer investigations were not pursued. Therefore in this study, firstly interactions of amodiaquine with human AChE and BChE were investigated and secondly with nerve agents in regard to its potential as a non-oxime reactivator. All data was determined spectrophotometrically by a modified Ellman assay. Amodiaquine is a reversible inhibitor of human cholinesterases with a higher inhibitory potency towards AChE (IC_{50} = 0.67 ± 0.02 μM) than BChE (IC_{50} = 81.28 ± 0.04 μM) (publication 2.2, Fig. 2). Determination of inhibition type was achieved by performing Michaelis-Menten kinetics and Lineweaver-Burk plot (publication 2.2, Fig. 3) and revealed a mixed competitive-non-competitive inhibition type of amodiaquine with AChE. As shown in figures 4 and 5 (publication 2.2) reactivation of tabun-inhibited cholinesterases by amodiaquine was insufficient, however, cyclosarin-, sarin-, and VX-inhibited cholinesterases were slowly and partially reactivated. Furthermore, prophylactic administration of amodiaquine prior to soman poisoning was tested in a dynamic model. Unfortunately, a protective effect or increased reactivation of cholinesterases was not observed (publication 2.2, Table 2). Thus, it can be concluded that administration of amodiaquine *in vivo* is unlikely due to its high inhibitory potency towards cholinesterases. However, the findings indicate that newly synthesized non-oxime reactivators could be based on amodiaquine's structure in order to increasing reactivation potency.

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Kinetic analysis of interactions of amodiaquine with human cholinesterases and organophosphorus compounds

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HIGHLIGHTS

- We investigated the interaction of human cholinesterases, OP nerve agents and amodiaquine.
- Amodiaquine is a potent inhibitor of human AChE and to less extent of human BChE.
- Amodiaquine resulted in slow and partial reactivation of sarin-, cyclosarin- and VX-inhibited AChE and BChE.
- Amodiaquine may be considered as a template for further non-oxime reactivators.

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ABSTRACT

Standard therapy of poisoning by organophosphorus compounds (OP) is a combined administration of an anti-muscarinic drug (*e.g.* atropine) and an oxime as reactivator of inhibited acetylcholinesterase (AChE). Limited efficacy of clinically used oximes against a variety of OPs was shown in numerous studies, calling for research on novel reactivators of OP-inhibited AChE. Recently, reactivation of OP-inhibited AChE by the antimalarial drug amodiaquine was reported. In the present study, amodiaquine and its interactions with human cholinesterases in presence or absence of OP nerve agents was investigated *in vitro*. Thereby, reversible inhibition of human cholinesterases by amodiaquine (AChE ≫ BChE) was observed. Additionally, a mixed competitive-non-competitive inhibition type of amodiaquine with human AChE was determined. Slow and partial reactivation of sarin-, cyclosarin- and VX-inhibited cholinesterases by amodiaquine was recorded, amodiaquine failed to reactivate tabun-inhibited human cholinesterases. Amodiaquine, being a potent, reversible AChE inhibitor, was tested for its potential benefit as a pretreatment to prevent complete irreversible AChE inhibition by the nerve agent soman. Hereby, amodiaquine failed to prevent phosphonylation and resulted only in a slight increase of AChE activity after removal of amodiaquine and soman. At present the molecular mechanism of amodiaquine-induced reactivation of OP-inhibited AChE is not known, nevertheless amodiaquine could be considered as a template for the design of more potent non-oxime reactivators.

1. Introduction

Since their discovery in the mid-1930s, highly toxic nerve agents have been deployed several times for example in civil war in Iraq, terrorist attacks in Tokyo or most recently in Syria (Eisenkraft et al., 2014; Macilwain, 1993; Nagao et al., 1997; UN Mission to Investigate Allegations of the Use of Chemical Weapons in the Syrian Arab Republic, 2013). Still, large stock piles of nerve agents are available despite being banned by the Chemical Weapons Convention (Organisation for the Prohibition of Chemical Weapons, 1997) and easy access to open source literature on synthetic routes (Holmstedt, 1951) are reasons why nerve agents still pose an ongoing threat.

Belonging to the superclass of organophosphorus compounds (OP), nerve agents exert their acute toxicity through irreversible inhibition of acetylcholinesterase (AChE; EC 3.1.1.7) by phosphylation (denotes phosphorylation and phosphonylation) of the serine hydroxyl group in its catalytic site (Marrs, 1993). Inhibited AChE is incapable of hydrolyzing ACh resulting in an excess of ACh at muscarinic (bradycardia, diarrhea, profuse sweating, salvation, miosis, bronchorrhoea, bronchoconstriction) and nicotinic (convulsions, muscle fasciculation, muscle dysfunction) receptors,

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eventually resulting in death by respiratory failure (Grob, 1956; Holmstedt, 1959). The current standard therapy consists of administration of a muscarinic antagonist (*e.g.* atropine) and an oxime that functions as a reactivator of inhibited AChE (Lee, 2003; Thiermann et al., 2013; Volans, 1996). Today, obidoxime, pralidoxime (2-PAM), and trimedoxime (TMB-4) are used clinically in OP poisoning. Limited efficacy of atropine-oxime combinations as standard treatment against different nerve agents, *e.g.* soman, tabun, and cyclosarin, has been shown in numerous *in vitro* and *in vivo* studies (Dawson, 1994; Eyer et al., 2007; Lundy et al., 1992; Marrs et al., 2006; Worek and Thiermann, 2013). Therefore, a vast number of novel oximes have been synthesized by various research groups so as to find more effective reactivators, mainly focusing on oximes that are effective against reactivation-resistant OP-AChE complexes or a broad-spectrum of OP nerve agents (Bismuth et al., 1992; Eyer and Worek, 2007; Hobbiger, 1963). Structure-activity studies identified a small number of promising bispyridinium oximes (Gray, 1984; Musilek et al., 2011; Schoene, 1980; Worek et al., 2012). However, despite extensive research for decades, no oxime that overcomes mentioned disadvantages has been discovered.

Consequently, research started focusing on non-oxime reactivators, which have preferential low toxicity, high blood-brain barrier penetration, efficacy against reactivation-resistant OP-AChE complexes and a broad-spectrum against different OP nerve agents. To identify a suitable non-oxime reactivator, Bhattacharjee and co-workers adopted an *in silico* strategy of pharmacophore modeling (Bhattacharjee et al., 2015, 2012; Leach et al., 2010) and used it for virtual screening databases. During the process several non-oxime reactivators were discovered with one reactivator being equally efficient as 2-PAM against DFP-induced neuropathology in an *in vivo* assay with guinea pigs (Bhattacharjee et al., 2015, 2012). Recently, Katz et al. (2015) performed *in silico* screening and investigated non-oxime reactivators, from which amodiaquine showed reactivating potency with paraoxon-inhibited AChE.

Amodiaquine has antimalarial and anti-inflammatory properties and is one of the few antimalarial drugs that are effective against the severe malaria tropica (Love et al., 1953; Mackenzie, 1983; Pomeroy et al., 1959). Numerous studies were performed investigating amodiaquine's mode of action, mainly focusing on its side effects and revealing that high doses of amodiaquine over a longer period cause hepatotoxicity and agranulocytosis, therefore prophylactic administration is impossible (Booth et al., 1967; Glick, 1957; Hatton et al., 1986; Neftel et al., 1986). Our investigation focused on interactions between amodiaquine and human AChE and butyrylcholinesterase (BChE) in presence and absence of OP nerve agents *in vitro* in order to evaluate the potential of amodiaquine as non-oxime reactivator.

2. Materials and methods

2.1. Materials

Acetylthiocholine iodide (ATCh), amodiaquine dihydrochloride dihydrate (AQ), *S*-butyrylthiocholine iodide (BTCh), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent), isolated human butyrylcholinesterase (BChE, EC 3.1.1.8), and tris(hydroxymethyl) aminomethane (Tris) were purchased from Sigma–Aldrich (Taufkirchen, Germany). Disodium hydrogenphosphate dihydrate, hydrochloride acid and potassium dihydrogenphosphate were obtained from Carl Roth (Karlsruhe, Germany). HI-6 dichloride monohydrate was provided by Dr. Clement (Defence Research Establishment Suffield, Ralston, Alberta, Canada). Millex®-GS 0.22 μM particle filters were supplied by Millipore (Eschborn, Germany).

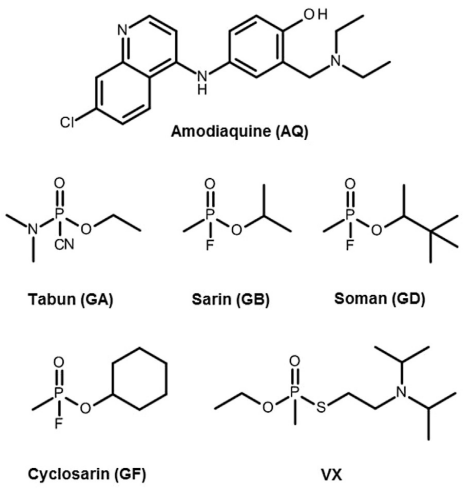

Fig. 1. Chemical structures of amodiaquine and organophosphorus compounds.

O-Ethyl-*S*-2-diisopropylaminoethylmethylphosphonothiolate (VX), tabun (GA), sarin (GB), cyclosarin (GF), and soman (GD) (>95% by GC–MS, ^{1}H NMR and ^{31}P NMR) (Fig. 1) were made available by the German Ministry of Defence (Bonn, Germany). All other chemicals were from Merck Eurolab GmbH (Darmstadt, Germany) at the purest grade available.

Stock solutions of tabun, sarin, cyclosarin, soman and VX (0.1% v/v) were prepared in acetonitrile, stored at room temperature, and diluted appropriately in either phosphate (0.1 M, pH 7.4) or Tris–HCl buffer (0.1 M, pH 7.4) depending on further experiments. Amodiaquine stock solution (50 mM) was prepared in distilled water, stored at −80 °C and diluted as required in distilled water prior to use.

2.2. Preparation of hemoglobin-free erythrocyte ghosts

Hemoglobin-free erythrocyte ghosts served as source of human erythrocyte acetylcholinesterase (AChE) and were prepared according to Dodge et al., 1963 with minor modifications (Worek et al., 2002). Briefly, heparinized human whole blood (donated by AB and FW) was centrifuged (3000 × *g*, 10 min) for plasma removal. The erythrocytes were washed three times with two volumes of phosphate buffer (0.1 M, pH 7.4). In order to facilitate hemolysis, the packed erythrocytes were diluted in 20 volumes of hypotonic phosphate buffer (6.7 mM, pH 7.4) and centrifuged at 50,000 × *g* (30 min, 4 °C). After two additional washing cycles the pellet was re-suspended in phosphate buffer (0.1 M, pH 7.4) and the initial AChE activity was adjusted. Aliquots of erythrocyte ghosts were stored at −80 °C. Prior to use, thawed erythrocyte ghosts were homogenized on ice with a Sonoplus HD 2070 ultrasonic homogenisator (Bandelin electronic, Berlin, Germany) twice for 5 s with a 20 s interval in between to achieve a homogenous matrix for kinetic studies.

2.3. Inhibition of AChE and BChE by amodiaquine

AChE or BChE and amodiaquine (0.1–500 μM) were added to polystyrol cuvettes containing DTNB (0.5 mM) as chromogen and ATCh (0.71 mM) or BTCh (1.58 mM) as substrate in 0.1 M phosphate buffer. Enzyme activities were determined spectrophotometrically (Cary 50 Bio, Varian, Darmstadt) at 412 nm for 1 min with a

modified Ellman assay (Ellman et al., 1961; Eyer et al., 2003; Worek et al., 1999). As a correction, measurements were performed against a reference cuvette lacking enzymes. Experiments were performed in duplicate and data were analyzed by non-linear regression. All experiments were performed at 37 °C and given concentrations refer to final concentrations.

2.4. Calculation of IC_{50}

IC_{50} values were calculated from semi-logarithmic plots of amodiaquine concentration versus enzyme activity (AChE or BChE).

2.5. Reversibility of AChE inhibition by amodiaquine

Investigation of reversibility of AChE inhibition by amodiaquine was carried out as followed: 200 μL AChE (37 °C) were incubated with 2 μL amodiaquine (5 μM f.c.) or distilled water as control. After 5 min, an aliquot (50 μL) was transferred into 0.1 M phosphate buffer (15 mL; 37 °C), mixed and incubated for 5 min. Afterwards, 3 mL incubate were transferred into a polystyrol cuvette containing DTNB (0.3 mM) and ATCh (0.45 mM) and the activity was measured at 412 nm for 3 min.

2.6. Determination of inhibition type of amodiaquine

To determine the inhibition type of amodiaquine, various ATCh concentrations (5–200 μM) in absence or presence of amodiaquine (0, 50, 100, and 200 nM) were added to polystyrol cuvettes (37 °C) containing AChE and DTNB (0.3 mM) and AChE activity was measured at 412 nm for 2 min. Experiments were performed in duplicate, recorded AChE activity was plotted *vs.* ATCh concentrations (Michaelis–Menten plot) and analyzed by non-linear regression. Linearization of data by plotting 1/v *vs.* 1/[S] was performed to obtain Lineweaver–Burk plots. Kinetic constants K_m and v_{max} were determined and used for distinguishing amodiaquine's inhibition type. Furthermore, two secondary re-plots of the Lineweaver–Burk plot were generated by plotting slope and ordinate intercepts of these lines *vs.* ligand concentration. From X-intercepts of straight lines, inhibition constants k_{ic} (slope *vs.* inhibitor concentration) and k_{iu} (ordinate *vs.* inhibitor concentration) were determined and α was calculated from ratio (k_{iu}/k_{ic}) indicating the inhibitor's influence on binding affinity of enzyme to substrate (Bisswanger, 1979).

2.7. Reactivation kinetics of OP-inhibited AChE or BChE by amodiaquine

OP-inhibited enzymes were prepared by adding a small volume (<1% v/v) of tabun, sarin, cyclosarin, and VX to AChE or BChE, followed by incubation for 15 min at 37 °C in order to achieve an inhibition of >95% of control activity. For removal of excess OP, inhibited samples were dialyzed in phosphate buffer (0.1 M, pH 7.4) overnight at 4 °C and residual enzyme activity was measured by incubation of OP-treated and control enzyme (30 min, 37 °C). Until use, aliquots were stored at −80 °C.

150 μL OP-treated enzyme was mixed with equal volume of phosphate buffer (0.1 M, pH 7.4) (containing 0.2% gelatin for stabilizing AChE) and at $t = 0$, 3 μL amodiaquine were added to initiate reactivation. After specified time intervals ($t = 2$–60 min), aliquots were transferred to cuvettes containing phosphate buffer, DTNB (0.3 mM) and ATCh (0.45 mM) or BTCh (1 mM) for measurement of enzyme activity at 412 nm for 3 min. Three different amodiaquine concentrations (1, 10, and 100 μM f.c.) were used in the experiments ($n = 2$ independent experiments). % reactivation was calculated by applying Eq. (1)

$$\%\text{reac} = \frac{(A_1 - A_i)}{(A_0 - A_i)} \quad (1)$$

with A_0 control enzyme activity, A_i OP-inhibited enzyme activity, and A_1 amodiaquine reactivated enzyme activity. The first order reactivation rate constants (k_{obs}) were determined by non-linear regression analysis.

2.8. Reactivation kinetics of OP-inhibited AChE by amodiaquine and HI-6

OP-inhibited AChE was mixed with equal volume of phosphate buffer (0.1 M, pH 7.4) containing 0.2% gelatin, HI-6 (50 μM) and amodiaquine (10 μM, $t = 0$) were added to initiate reactivation. After specified time intervals ($t = 2$–60 min), aliquots were transferred to cuvettes containing phosphate buffer, DTNB (0.3 mM) and ATCh (0.45 mM) for measurement of enzyme activity at 412 nm for 1 min. OP-inhibited enzyme activity and enzyme activity after time-dependent reactivation was referred to control enzyme activity and % reactivation was calculated thereof. Experiments were performed in duplicate.

2.9. Determination of detoxification constants

To investigate detoxification kinetics of different OPs by amodiaquine, an AChE inhibition assay was used. Therefore, 150 μL amodiaquine were mixed with 75 μL Tris–HCl (0.1 M, pH 7.4) and 75 μM OP (235 nM sarin, 40 nM cyclosarin, and 80 nM VX f.c.). Amodiaquine concentration was 0.5 equimolar of the respective OP concentration. After specified time intervals ($t = 0$–60 min), aliquots were transferred to pre-heated cuvettes (37 °C) containing phosphate buffer, DTNB (0.3 mM) and ATCh (0.75 mM) for recording the progressive inhibition of AChE at 436 nm for 5 min ($n = 4$).

Detoxification constants were determined according to Bierwisch et al. (2014). In brief, first order inhibition rate constant was calculated by analyzing recorded inhibition curves by non-linear regression analysis. Inhibition rate constants were plotted *vs.* incubation time. By fitting the resulting decay curves, first order detoxification constants k_1 were obtained. k_1 was corrected for the spontaneous hydrolysis of the corresponding OP. Finally, half-lives were calculated on the basis of Eq. (2).

$$t_{1/2} = \frac{\ln 2}{k_1} \quad (2)$$

2.10. Dynamic model—general experimental procedure

For the online analysis of AChE activity, the well published dynamic model (Eckert et al., 2006a,b) was used. In brief, 80 μL human erythrocytes were diluted in 5 mL phosphate buffer (0.1 M, pH 7.4) and subsequently 4.5 mL were layered onto a particle filter (Millex®-GS, 0.22 μM, Ø 33 mm) with a peristaltic pump within 14 min. The AChE loaded enzyme reactor was submerged into a water bath (`37 °C, $t = 0$ min) and perfused with phosphate buffer (0.1 M, pH 7.4). Continuous perfusion of enzyme reactor with ATCh (0.45 mM), DTNB (0.3 mM), and phosphate buffer (0.1 M, pH 7.4) was started at $t = 15$ min to measure control AChE activity ($t = 30$ min). The total flow rate through the reactor was 0.5 mL/min with the effluent passing a photometer (LaChrom L-7420 UV–vis detector, Merck, Darmstadt, Germany) set at 470 nm. In 1.6 s intervals absorbance values were collected. The setup of the perfusion system consist of two HPLC pumps with integrated quaternary low-pressure gradient formers

(LaChrom L-1700, Merck), controlled by D-7000HPLC system manager software (Vers. 4.1, Merck).

2.11. Perfusion protocol for inhibition and reactivation of AChE by soman and amodiaquine

- Inhibition by GD: The enzyme reactor was perfused with 22 nM GD from 60 to 90 min.
- Perfusion with amodiaquine and GD: The enzyme reactor was perfused with 100 nM amodiaquine (t = 30 – 100 min) and with 22 nM GD from 60 to 90 min.

The recorded absorbance values were referred to the maximum AChE activity in order to calculate the % inhibition and % reactivation. Data are presented as means of four individual experiments per group.

2.12. Data analysis

Kinetic constants were calculated from experimental data by linear or non-linear regression analysis using Prism 5.04 (GraphPad Software, San Diego, CA, USA). Data are presented as means ± SD and number of experiments n.

3. Results

3.1. Inhibition of AChE and BChE by amodiaquine

Enzyme activities were measured with different amodiaquine concentrations to determine the inhibition of cholinesterases. Amodiaquine resulted in complete inhibition of AChE activity already at 20 μM while only an incomplete inhibition of BChE was recorded with 500 μM amodiaquine (Fig. 2). This difference is reflected by the calculated IC_{50} values of 669 nM with AChE (R^2 = 0.99) and 81 μM with BChE (R^2 = 0.94).

The reversibility of AChE inhibition by amodiaquine was investigated by inhibiting AChE with 5 μM amodiaquine followed by extensive dilution (300-fold) and determination of enzyme activity. A complete recovery of AChE activity was recorded indicating reversible inhibition by amodiaquine.

3.2. Determination of inhibition type of amodiaquine

The inhibition type of amodiaquine was investigated by analysis of AChE activities at different substrate and amodiaquine concentrations. As by definition, a classical non-competitive inhibitor has a decreasing v_{max} and constant K_m value by increasing inhibitor concentrations and an intersection point on the abscissa of the Lineweaver–Burk plot. Therefore, generated Michaelis–Menten and double reciprocal Lineweaver–Burk plots (Fig. 3) with an intersection within the second quadrant and to the left of the ordinate indicate that amodiaquine is a mixed competitive-non-competitive inhibitor of AChE. Additionally, k_{ic} and k_{iu} values were obtained from secondary re-plots of the Lineweaver–Burk plot and α calculated from the ratio of k_{iu}/k_{ic}, which was 1.45. An α value > 1 implies an intersection above the abscissa and also confirms a mixed competitive-non-competitive inhibition type of amodiaquine.

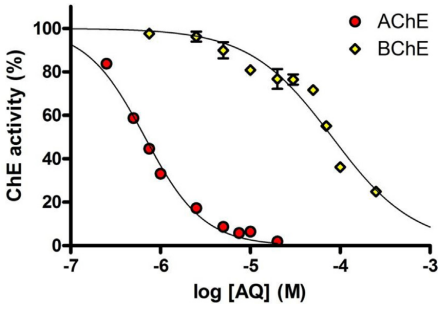

Fig. 2. Effect of amodiaquine on AChE and BChE activity. Various amodiaquine concentrations (0.1–500 μM) were added to cholinesterases and residual enzyme activity was measured. Data are shown as means ± SD (n = 2).

3.3. Reactivation kinetics of OP-inhibited AChE and BChE by amodiaquine

Reactivation of tabun-, sarin-, cyclosarin-, and VX-inhibited AChE and BChE by amodiaquine was determined with 1, 10 and 100 μM amodiaquine. The slow and partial reactivation of OP-inhibited AChE and BChE by amodiaquine decreased in the order VX > GF = GB while tabun-inhibited cholinesterases could not be reactivated at all (Fig. 4 and 5). Reactivation of BChE was strictly concentration-dependent while with AChE an increase of the amodiaquine concentration from 10 to 100 μM resulted in a decreased level of reactivation. This may be attributed to the high inhibitory potency of amodiaquine with AChE. In fact, the residual amodiquine concentration during the AChE assay (~0.6 μM at 100 μM incubation concentration) results in substantial AChE inhibition (cf. Fig. 2).

Additionally, the potential synergistic effect of amodiaquine and HI-6 was investigated with OP-inhibited AChE. In presence of 10 μM amodiaquine, the velocity and extent of reactivation by 50 μM HI-6 was reduced moderately (data not shown).

3.4. Determination of detoxification constants

Amodiaquine was incubated with a twofold excess of OP and the time-dependent effect on AChE inhibition was determined. From these data, calculation of detoxification constants and half-lives were performed. After correction for spontaneous hydrolysis no detoxification of tabun, sarin, cyclosarin and VX by amodiaquine occurred.

3.5. Inhibition and reactivation of AChE by soman and amodiaquine

The ability of amodiaquine to protect human AChE from irreversible inhibition by soman was investigated with the dynamic model with real-time determination of enzyme activity. Perfusion of enzyme reactors with 22 nM soman (t = 60–90 min) resulted in rapid and virtually complete AChE inhibition (Fig. 6 and Table 2). Perfusion of enzyme reactors with 100 nM amodiaquine in the absence of soman (t = 30–60 min) resulted in an AChE inhibition by 80% (Fig. 6). Additional perfusion with soman (t = 60–90 min) induced complete inhibition (Table 2). Termination of amodiaquine perfusion at t = 100 min resulted in a small increase of AChE activity which was absent in the soman only group (Fig. 6 and Table 2).

4. Discussion

The recent observation of partial reactivation of paraoxon-inhibited AChE by the non-oxime amodiaquine (Katz et al., 2015) prompted us to test the ability of amodiaquine to reactivate nerve agent-inhibited human AChE and BChE and to investigate a potential inhibitory potential of this compound with human cholinesterases.

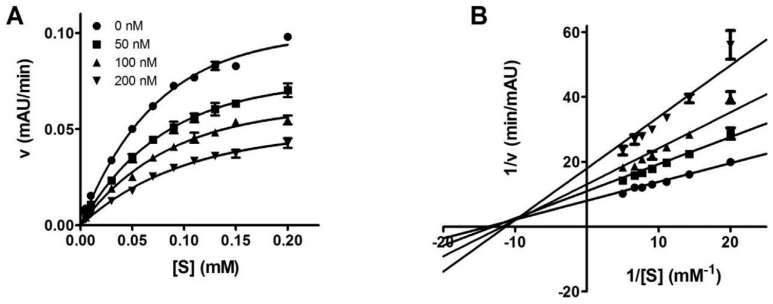

Fig. 3. Determination of inhibition type of amodiaquine by measuring substrate concentration-dependent curves of AChE activities with different amodiaquine concentrations. The generated data were analyzed and plotted according to Michaelis–Menten (A) and Lineweaver–Burk (B). Experiments were performed in duplicate and means ± SD are shown.

Antimalarial drugs, *e.g.* chloroquine, quinacrine, primaquine (Wright and Sabine, 1948) and amodiaquine (Go et al., 1981) are inhibitors of cholinesterases. The inhibitory activity of amodiaquine was related to binding of its phenolic hydroxyl and protonated diethylamino moiety or protonated quinolyl nitrogen in the esteric and anionic site of AChE (Go et al., 1981). AChE activity is fully regained after inhibition by amodiaquine, thus being a reversible inhibitor. Calculated IC_{50} values characterize amodiaquine as a highly potent inhibitor of human AChE whereas its inhibitory potency was low with human BChE (Fig. 2). Poor fitting of amodiaquine into the BChE binding site may explain its lower inhibitory potency with BChE. Due to a different composition of aromatic residues in human AChE and BChE, BChE has a larger available space in its binding site, thus preferring binding of larger substrates and inhibitors to its esteric or anionic site than human AChE.

Generated Michaelis–Menten and Lineweaver–Burk plots and thereof calculated v_{max}, and K_m values (Fig. 3) were used to identify the inhibition type of amodiaquine. Decreasing K_m and v_{max} values in Michaelis–Menten plot, an intersection within the second quadrant, above the abscissa with an $\alpha > 1$ and to the left of the ordinate of the Lineweaver–Burk plot indicate that amodiaquine is as a mixed competitive-non-competitive inhibitor of human AChE. The obtained mixed competitive-non-competitive inhibition type of amodiaquine is in contrast to findings of Go et al. (1981), who determined a competitive inhibition type for amodiaquine with electric eel AChE.

In previous studies, therapeutic plasma levels of amodiaquine in humans were determined to be less than 0.1 μM (Liu et al., 2014; Orrell et al., 2008) which may give an explanation of the absence of cholinergic side effects during the therapy with this antimalarial drug.

Sarin-, cyclosarin- and VX-inhibited human AChE and BChE was partially reactivated by amodiaquine, however at a slow rate (Fig. 4 and 5). In comparison to the bispyridinium oxime HI-6 amodiaquine was a substantially less potent reactivator of nerve agent-inhibited human AChE (Table 1). The high intrinsic inhibitory potency of amodiaquine with human AChE does not allow the use of high amodiaquine concentrations. In fact, incubation of OP-inhibited AChE with 100 μM amodiaquine

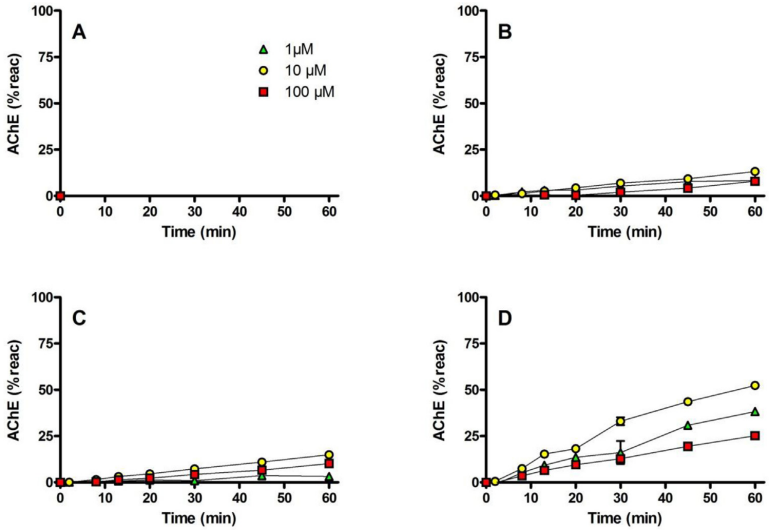

Fig. 4. Kinetics of reactivation of AChE inhibited by tabun (A), sarin (B), cyclosarin (C) and VX (D) by various amodiaquine concentrations. The AChE activities are expressed as % reactivation. Data are shown as means ± SD ($n = 2$).

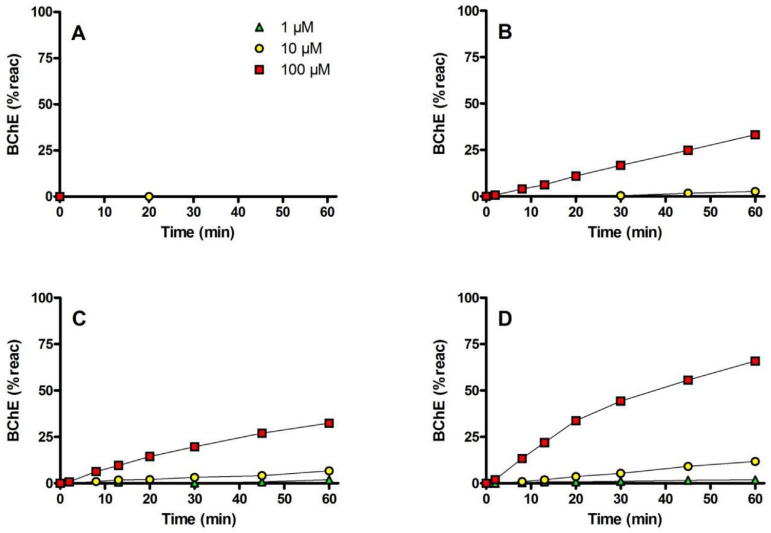

Fig. 5. Kinetics of reactivation of BChE inhibited by tabun (A), sarin (B), cyclosarin (C) and VX (D) by various amodiaquine concentrations. The BChE activities are expressed as % reactivation. Data are shown as means ± SD ($n = 2$).

resulted in a reduced apparent reactivation which was potentially due to the inhibition of reactivated AChE by the residual amodiaquine concentration (~0.6 µM) during the AChE assay. In comparison, a concentration-dependent increase of reactivation was recorded with OP-inhibited BChE, most probably due to the low inhibitory activity of amodiaquine with human BChE.

The underlying mechanism of reactivation of OP-inhibited cholinesterases by amodiaquine has not been discovered yet. One conceivable mechanism could be interaction of amodiaquine within the active site and therefore being in close proximity to the covalently bound serine, which is reactivated. Otherwise amodiaquine, as a mixed competetive-non-competitive inhibitor, could also interact with the peripheral anionic site (PAS) and generate conditions in the active site were cleavage of OP-residue is preferential and reactivation can occur.

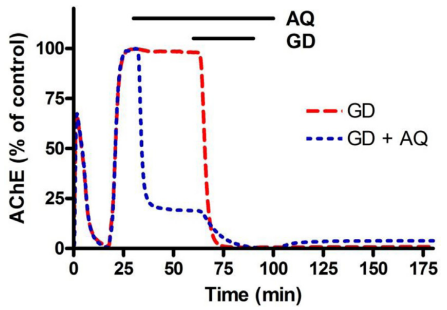

Fig. 6. Inhibition and reactivation of human AChE with soman in presence or absence of amodiaquine. Time-course of AChE activity following inhibition with 22 nM soman (GD; $t = 60$–90 min) or pre-treatment of 100 nM amodiaquine starting at $t = 30$ min followed by 22 nM GD ($t = 60$–90 min). Amodiaquine perfusion was terminated at $t = 100$ min (indicated by horizontal bars). Data are shown as means of 4 experiments as % of control.

Simultaneous incubation of OP-inhibited AChE with amodiaquine (10 µM) and HI-6 (50 µM) resulted in a moderate decrease of HI-6-induced reactivation. This may be attributed to a competition of the highly potent AChE inhibitor amodiaquine with the oxime.

The ability of amodiaquine to reactivate nerve agent-inhibited cholinesterases prompted us to investigate if amodiaquine could detoxify these agents by binding or hydrolysis as was shown previously with other small molecules (Bierwisch et al., 2014). Unfortunately, incubation of amodiaquine with sarin, cyclosarin or VX did not result in a reduction of AChE inhibition which indicates that amodiaquine has no potential to serve as a small molecule scavenger.

Previous studies showed that pretreatment of human AChE with carbamates and the reversible AChE inhibitor huperzine A protects the enzyme to some degree from irreversible phosphylation by soman (Eckert et al., 2007, 2006b; Herkert et al., 2011, 2008). Hence, it was tempting to investigate whether the potent AChE inhibitor amodiaquine would show a comparable effect. Utilizing a dynamic model with real-time determination of AChE activity allowed the online recording of inhibition and reactivation kinetics in the presence of amodiaquine and soman. Preincubation of AChE with amodiaquine (100 nM) resulted in approx. 80%

Table 1
Reactivation of OP-inhibited AChE or BChE by amodiaquine. Reactivation rate constants (k_{obs}) were determined by non-linear regression analysis. Data are given as means ± SD ($n = 2$). Ø calculation of reactivation constant not possible due to inadequate reactivation of inhibited AChE. For comparison, the corresponding k_{obs} values for HI-6 were included.

	k_{obs} [min^{-1}]			
	AChE		BChE	
	10 µM AQ	10 µM HI-6[a]	100 µM AQ	100 µM HI-6[b]
GA	Ø	Ø	Ø	0.0004
GB	Ø	0.1128	0.0063	0.0265
GF	Ø	0.2068	0.0069	0.1273
VX	0.0124	0.1126	0.0187	0.0190

[a] from Worek et al. (2004).
[b] from Aurbek et al. (2009).

Table 2
Inhibition of human AChE by amodiaquine and soman and reactivation after washout of inhibitors.

	k_1 [min^{-1}]	max. inhib (%)	max. reac (%)
GD	0.54 ± 0.11	99.43 ± 0.61	1.14 ± 0.74
AQ + GD	0.11 ± 0.02	99.85 ± 1.85	5.43 ± 1.88

The experiments were performed with 22 nM GD (t = 60–90 min) or pre-treatment of 100 nM amodiaquine (t = 30–100 min) followed by 22 nM GD (t = 60–90 min). k_1 denotes the first order inhibition rate constant, max. inhib the maximum AChE inhibition, max. reac. the maximum reactivation at the end of the experiment (t = 180 min).

inhibition (Fig. 6), slowed down the subsequent inhibition by soman (22 nM) but could not prevent complete inhibition of the enzyme (Table 2). However, pretreatment of AChE with amodiaquine resulted in a slight but significant recovery of AChE activity after termination of perfusion with amodiaquine and soman which indicates the protection of a small portion of AChE from phosphylation by soman. In comparison to huperzine A (33.7%), as well as the carbamates pyridostigmine and physostigmine, the extent of final recovery of AChE activity was markedly lower with amodiaquine (5.4%) at a comparable level of initial inhibition by the reversible inhibitors (Eckert et al., 2007; Herkert et al., 2011, 2008). These data indicate that it is unlikely to consider amodiaquine as a promising candidate for the pretreatment of anticipated exposure to highly toxic nerve agents like soman.

In conclusion, the non-oxime amodiaquine proved to be a reactivator of a broad spectrum of OP nerve agents although the exact mechanism of action is not known. The high inhibitory potency of amodiaquine towards human AChE is the major limitation for its use *in vivo*. Nevertheless, the unexpected potential of amodiaquine to reactivate OP-inhibited AChE makes it a suitable template for the design of more effective non-oxime reactivators with less intrinsic inhibitory activity towards human AChE.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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References

Aurbek, N., Thiermann, H., Eyer, F., Eyer, P., Worek, F., 2009. Suitability of human butyrylcholinesterase as therapeutic marker and pseudo catalytic scavenger in organophosphate poisoning: a kinetic analysis. Toxicology 259, 133–139. doi:http://dx.doi.org/10.1016/j.tox.2009.02.014.

Bhattacharjee, A.K., Marek, E., Le, H.T., Gordon, R.K., 2012. Discovery of non-oxime reactivators using an in silico pharmacophore model of oxime reactivators of OP-inhibited acetylcholinesterase. Eur. J. Med. Chem. 49, 229–238. doi:http://dx.doi.org/10.1016/j.ejmech.2012.01.016.

Bhattacharjee, A.K., Marek, E., Le, H.T., Ratcliffe, R., DeMar, J.C., Pervitsky, D., Gordon, R.K., 2015. Discovery of non-oxime reactivators using an in silico pharmacophore model of reactivators for DFP-inhibited acetylcholinesterase. Eur. J. Med. Chem. 90, 209–220. doi:http://dx.doi.org/10.1016/j.ejmech.2014.11.013.

Bierwisch, A., Zengerle, M., Thiermann, H., Kubik, S., Worek, F., 2014. Detoxification of alkyl methylphosphonofluoridates by an oxime-substituted β-cyclodextrin—an in vitro structure-activity study. Toxicol. Lett. 224, 209–214. doi:http://dx.doi.org/10.1016/j.toxlet.2013.10.024.

Bismuth, C., Inns, R.H., Marrs, T.C., 1992. Efficacy, toxicity and clinical use of oximes in anticholinesterase poisoning.. In: Ballantyne, B., Marrs, T.C. (Eds.), Clinical and Experimental Toxicology of Organophosphates and Carbamates. Butterworth & Heinemann, Oxford, pp. 555–577.

Bisswanger, H., 1979. Enzymkinetik. Theorie und Methoden der Enzymkinetik. Verlag Chemie Weinheim, Deerfield Beach (Florida) Basel.

Booth, K., Larkin, K., Maddocks, I., 1967. Agranulocytosis coincident with amodiaquine therapy. Br. Med. J. 3, 32–33.

Dawson, R.M., 1994. Review of oximes available for treatment of nerve agent poisoning. J. Appl. Toxicol. 14, 317–331.

Dodge, J.T., Mitchell, C., Hanahan, D.J., 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Arch. Biochem. Biophys. 100, 119–130.

Eckert, S., Eyer, P., Mückter, H., Worek, F., 2006a. Development of a dynamic model for real-time determination of membrane-bound acetylcholinesterase activity upon perfusion with inhibitors and reactivators. Biochem. Pharmacol. 72, 358–365. doi:http://dx.doi.org/10.1016/j.bcp.2006.04.016.

Eckert, S., Eyer, P., Mückter, H., Worek, F., 2006b. Kinetic analysis of the protection afforded by reversible inhibitors against irreversible inhibition of acetylcholinesterase by highly toxic organophosphorus compounds. Biochem. Pharmacol. 72, 344–357. doi:http://dx.doi.org/10.1016/j.bcp.2006.04.015.

Eckert, S., Eyer, P., Worek, F., 2007. Reversible inhibition of acetylcholinesterase by carbamates or huperzine A increases residual activity of the enzyme upon soman challenge. Toxicology 233, 180–186. doi:http://dx.doi.org/10.1016/j.tox.2006.09.012.

Eisenkraft, A., Gilburd, D., Kassirer, M., Kreiss, Y., 2014. What can we learn on medical preparedness from the use of chemical agents against civilians in Syria? Am. J. Emerg. Med. 32, 186. doi:http://dx.doi.org/10.1016/j.ajem.2013.11.005.

Ellman, G.L., Courtney, K.D., Andres, V., Feather-Stone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Eyer, P., Szinicz, L., Thiermann, H., Worek, F., Zilker, T., 2007. Testing of antidotes for organophosphorus compounds: experimental procedures and clinical reality. Toxicology 233, 108–119. doi:http://dx.doi.org/10.1016/j.tox.2006.08.033.

Eyer, P., Worek, F., 2007. Oximes. In: Marrs, T.C., Maynard, R.L., Sidell, F.R. (Eds.), Chemical Warfare Agents: Toxicology and Treatment. John Wiley & Sons Ltd., Chichester, pp. 302–329.

Eyer, P., Worek, F., Kiderlen, D., Sinko, G., Stuglin, A., Simeon-Rudolf, V., Reiner, E., 2003. Molar absorption coefficients for the reduced Ellman reagent: reassessment. Anal. Biochem. 312, 224–227.

Glick, L., 1957. Fatal agranulocytosis during treatment with a amodiaquine. Br. Med. J. 1, 932.

Go, M.L., Ngiam, T.L., Wan, A.S., 1981. Investigation of the anti-acetylcholinesterase activities of the antimalarial agent, amodiaquine, and related compounds. Southeast Asian J. Trop. Med. Public Health 12, 37–41.

Gray, A.P., 1984. Design and structure-activity relationships of antidotes to organophosphorus anticholinesterase agents. Drug. Metab. Rev. 15, 557–589. doi:http://dx.doi.org/10.3109/03602538409029973.

Grob, D., 1956. The manifestations and treatment of poisoning due to nerve gas and other organic phosphate anticholinesterase compounds. AMA Arch. Intern. Med. 98, 221–239.

Hatton, C.S., Peto, T.E., Bunch, C., Pasvol, G., Russell, S.J., Singer, C.R., Edwards, G., Winstanley, P., 1986. Frequency of severe neutropenia associated with amodiaquine prophylaxis against malaria. Lancet Lond. Engl. 1, 411–414.

Herkert, N.M., Eckert, S., Eyer, P., Bumm, R., Weber, G., Thiermann, H., Worek, F., 2008. Identical kinetics of human erythrocyte and muscle acetylcholinesterase with respect to carbamate pre-treatment, residual activity upon soman challenge and spontaneous reactivation after withdrawal of the inhibitors. Toxicology 246, 188–192. doi:http://dx.doi.org/10.1016/j.tox.2008.01.010.

Herkert, N.M., Thiermann, H., Worek, F., 2011. In vitro kinetic interactions of pyridostigmine, physostigmine and soman with erythrocyte and muscle acetylcholinesterase from different species. Toxicol. Lett. 206, 41–46. doi:http://dx.doi.org/10.1016/j.toxlet.2011.03.004.

Hobbiger, F., 1963. Reactivation of phosphorylated acetylcholinesterase. In: Koelle, G.B. (Ed.), Cholinesterases and Anticholinesterase Agents. Springer, Berlin, pp. 921–988.

Holmstedt, B., 1959. Pharmacology of organophosphorus cholinesterase inhibitors. Pharmacol. Rev. 11, 567–688.

Holmstedt, B., 1951. Synthesis and pharmacology of dimethylamido-ethoxy-phosphoryl cyanide (tabun) together with a description of some allied anticholinesterase compounds containing the N—P bond. Acta Physiol. Scand. Suppl. 25, 12–120.

Katz, F.S., Pecic, S., Tran, T.H., Trakht, I., Schneider, L., Zhu, Z., Ton-That, L., Luzac, M., Zlatanic, V., Damera, S., Macdonald, J., Landry, D.W., Tong, L., Stojanovic, M.N., 2015. Discovery of new classes of compounds that reactivate acetylcholinesterase inhibited by organophosphates. Chembiochem 16, 2205–2215. doi:http://dx.doi.org/10.1002/cbic.201500348.

Leach, A.R., Gillet, V.J., Lewis, R.A., Taylor, R., 2010. Three-dimensional pharmacophore methods in drug discovery. J. Med. Chem. 53, 539–558. doi:http://dx.doi.org/10.1021/jm900817u.

Lee, E.C., 2003. Clinical manifestations of sarin nerve gas exposure. JAMA 290, 659–662. doi:http://dx.doi.org/10.1001/jama.290.5.659.

Liu, Y., Hu, C., Liu, G., Jia, J., Yu, C., Zhu, J., Zheng, Q., Zhang, K.E., 2014. A replicate designed bioequivalence study to compare two fixed-dose combination products of artesunate and amodiaquine in healthy chinese volunteers. Antimicrob. Agents Chemother. 58, 6009–6015. doi:http://dx.doi.org/10.1128/AAC.;1;02617-14.

Love, J., Foulk, R., Williams, R.G.W., Mitchell, R.B., 1953. Evaluation of amodiaquin (camoquin) in the treatment of relapsing vivax malaria. Am. J. Med. Sci. 225, 26–33.

Lundy, P.M., Hansen, A.S., Hand, B.T., Boulet, C.A., 1992. Comparison of several oximes against poisoning by soman tabun and GF. Toxicology 72, 99–105.

Macilwain, C., 1993. Study proves Iraq used nerve gas. Nature 363, 3. doi:http://dx.doi.org/10.1038/363003b0.

Mackenzie, A.H., 1983. Antimalarial drugs for rheumatoid arthritis. Am. J. Med. 75, 48–58.

Marrs, T.C., 1993. Organophosphate poisoning. Pharmacol. Ther. 58, 51–66.

Marrs, T.C., Rice, P., Vale, J.A., 2006. The role of oximes in the treatment of nerve agent poisoning in civilian casualties. Toxicol. Rev. 25, 297–323.

Musilek, K., Dolezal, M., Gunn-Moore, F., Kuca, K., 2011. Design, evaluation and structure-activity relationship studies of the AChE reactivators against organophosphorus pesticides. Med. Res. Rev. 31, 548–575. doi:http://dx.doi.org/10.1002/med.20192.

Nagao, M., Takatori, T., Matsuda, Y., Nakajima, M., Iwase, H., Iwadate, K., 1997. Definitive evidence for the acute sarin poisoning diagnosis in the Tokyo subway. Toxicol. Appl. Pharmacol. 144, 198–203. doi:http://dx.doi.org/10.1006/taap.1997.8110.

Neftel, K.A., Woodtly, W., Schmid, M., Frick, P.G., Fehr, J., 1986. Amodiaquine induced agranulocytosis and liver damage. Br. Med. J. Clin. Res. Ed. 292, 721–723.

Organisation for the Prohibition of Chemical Weapons, 1997. Convention on the prohibition of the development, production, stockpiling and use of chemical weapons and on their destruction.

Orrell, C., Little, F., Smith, P., Folb, P., Taylor, W., Olliaro, P., Barnes, K.I., 2008. Pharmacokinetics and tolerability of artesunate and amodiaquine alone and in combination in healthy volunteers. Eur. J. Clin. Pharmacol. 64, 683–690. doi:http://dx.doi.org/10.1007/s00228-007-0452-8.

Pomeroy, H., Warren, C., Mills, D., Clark, G.M., 1959. The effect of amodiaquin (camoquin) on the course of rheumatoid arthritis. Arthritis Rheumatol. 2, 396–402.

Schoene, K., 1980. Pyridinium salts as organophosphate antagonists. Monogr. Neural Sci. 7, 85–98.

Thiermann, H., Worek, F., Kehe, K., 2013. Limitations and challenges in treatment of acute chemical warfare agent poisoning. Chem. Biol. Interact. 206, 435–443. doi:http://dx.doi.org/10.1016/j.cbi.2013.09.015.

UN Mission to Investigate Allegations of the Use of Chemical Weapons in the Syrian Arab Republic, 2013. Report on Allegations of the Use of Chemical Weapons in Ghouta Area of Damascus on 21 August 2013.

Volans, A.P., 1996. Sarin: guidelines on the management of victims of a nerve gas attack. J. Accid. Emerg. Med. 13, 202–206.

Worek, F., Mast, U., Kiderlen, D., Diepold, C., Eyer, P., 1999. Improved determination of acetylcholinesterase activity in human whole blood. Clin. Chim. Acta. 288, 73–90.

Worek, F., Reiter, G., Eyer, P., Szinicz, L., 2002. Reactivation kinetics of acetylcholinesterase from different species inhibited by highly toxic organophosphates. Arch. Toxicol. 76, 523–529. doi:http://dx.doi.org/10.1007/s00204-002-0375-1.

Worek, F., Thiermann, H., 2013. The value of novel oximes for treatment of poisoning by organophosphorus compounds. Pharmacol. Ther. 139, 249–259. doi:http://dx.doi.org/10.1016/j.pharmthera.2013.04.009.

Worek, F., Thiermann, H., Szinicz, L., Eyer, P., 2004. Kinetic analysis of interactions between human acetylcholinesterase, structurally different organophosphorus compounds and oximes. Biochem. Pharmacol. 68, 2237–2248. doi:http://dx.doi.org/10.1016/j.bcp.2004.07.038.

Worek, F., von der Wellen, J., Musilek, K., Kuca, K., Thiermann, H., 2012. Reactivation kinetics of a homologous series of bispyridinium bis-oximes with nerve agent-inhibited human acetylcholinesterase. Arch. Toxicol. 86, 1379–1386. doi:http://dx.doi.org/10.1007/s00204-012-0842-2.

Wright, C.I., Sabine, J.C., 1948. Cholinesterases of human erythrocytes and plasma and their inhibition by antimalarial drugs. J. Pharmacol. Exp. Ther. 93, 230–239.

3 Bibliography

1. Aldridge, N. & Reiner, E. *Enzyme inhibitors as substrates - interaction of esterases with esters of organophosphorus and carbamic acids.* (North-Holland Publishing Company, Amsterdam, London, 1972).
2. Ashani, Y., Bhattacharjee, A. K., Leader, H., Saxena, A. & Doctor, B. P. Inhibition of cholinesterases with cationic phosphonyl oximes highlights distinctive properties of the charged pyridine groups of quaternary oxime reactivators. *Biochem. Pharmacol.* **66,** 191–202 (2003).
3. Ashani, Y. & Pistinner, S. Estimation of the upper limit of human butyrylcholinesterase dose required for protection against organophosphates toxicity: a mathematically based toxicokinetic model. *Toxicol. Sci.* **77,** 358–367 (2004).
4. Aurbek, N., Thiermann, H., Eyer, F., Eyer, P. & Worek, F. Suitability of human butyrylcholinesterase as therapeutic marker and pseudo catalytic scavenger in organophosphate poisoning: a kinetic analysis. *Toxicology* **259,** 133–139 (2009).
5. Barak, D., Ordentlich, A., Kaplan, D., Barak, R., Mizrahi, D., Kronman, C., Segall, Y., Velan, B. & Shafferman, A. Evidence for P-N bond scission in phosphoroamidate nerve agent adducts of human acetylcholinesterase. *Biochemistry* **39,** 1156–1161 (2000).
6. Bedford, C. D., Howd, R. A., Dailey, O. D., Miller, A., Nolen, H. W., Kenley, R. A., Kern, J. R. & Winterle, J. S. Nonquaternary cholinesterase reactivators. 3. 3(5)-Substituted 1,2,4-oxadiazol-5(3)-aldoximes and 1,2,4-oxadiazole-5(3)-thiocarbohydroximates as reactivators of organophosphonate-inhibited eel and human acetylcholinesterase in vitro. *J. Med. Chem.* **29,** 2174–2183 (1986).
7. Bencsura, A., Enyedy, I. & Kovach, I. M. Origins and diversity of the aging reaction in phosphonate adducts of serine hydrolase enzymes: what characteristics of the active site do they probe? *Biochemistry* **34,** 8989–8999 (1995).
8. Benschop, H. P. & De Jong, L. P. Toxicokinetics of soman: species variation and stereospecificity in elimination pathways. *Neurosci. Biobehav. Rev.* **15,** 73–77 (1991).
9. Benschop, H. P. & Keijer, J. H. On the mechanism of ageing of phosphonylated cholinesterases. *Biochim. Biophys. Acta* **128,** 586–588 (1966).
10. Bhattacharjee, A. K., Marek, E., Le, H. T. & Gordon, R. K. Discovery of non-oxime reactivators using an in silico pharmacophore model of oxime reactivators of OP-inhibited acetylcholinesterase. *Eur. J. Med. Chem.* **49,** 229–238 (2012).
11. Bhattacharjee, A. K., Marek, E., Le, H. T., Ratcliffe, R., DeMar, J. C., Pervitsky, D. & Gordon, R. K. Discovery of non-oxime reactivators using an in silico pharmacophore model of reactivators for DFP-inhibited acetylcholinesterase. *Eur. J. Med. Chem.* **90,** 209–220 (2015).
12. Black, R. M., Clarke, R. J., Read, R. W. & Reid, M. T. Application of gas chromatography-mass spectrometry and gas chromatography-tandem mass spectrometry to the analysis of chemical warfare samples, found to contain residues of the nerve agent sarin, sulphur mustard and their degradation products. *J. Chromatogr. A* **662,** 301–321 (1994).
13. Brandhuber, F., Zengerle, M., Porwol, L., Bierwisch, A., Koller, M., Reiter, G., Worek, F. & Kubik, S. Tabun scavengers based on hydroxamic acid containing cyclodextrins. *Chem. Commun. (Camb.)* **49,** 3425–3427 (2013).
14. Brandhuber, F., Zengerle, M., Porwol, L., Tenberken, O., Thiermann, H., Worek, F., Kubik, S. & Reiter, G. Detoxification of tabun at physiological pH mediated by substituted β-cyclodextrin and glucose derivatives containing oxime groups. *Toxicology* **302,** 163–171 (2012).

15. Broomfield, C. A., Maxwell, D. M., Solana, R. P., Castro, C. A., Finger, A. V. & Lenz, D. E. Protection by butyrylcholinesterase against organophosphorus poisoning in nonhuman primates. *J. Pharmacol. Exp. Ther.* **259,** 633–638 (1991).

16. Cadieux, C. L., Wang, H., Zhang, Y., Koenig, J. A., Shih, T.-M., McDonough, J., Koh, J. & Cerasoli, D. Probing the activity of a non-oxime reactivator for acetylcholinesterase inhibited by organophosphorus nerve agents. *Chem. Biol. Interact. (in press)* (2016).

17. Cannard, K. The acute treatment of nerve agent exposure. *J. Neurol. Sci.* **249,** 86–94 (2006).

18. Cerasoli, D. M., Griffiths, E. M., Doctor, B. P., Saxena, A., Fedorko, J. M., Greig, N. H., Yu, Q. S., Huang, Y., Wilgus, H., Karatzas, C. N., Koplovitz, I. & Lenz, D. E. In vitro and in vivo characterization of recombinant human butyrylcholinesterase (Protexia) as a potential nerve agent bioscavenger. *Chem. Biol. Interact.* **157-158,** 363–365 (2005).

19. Cherny, I., Greisen, P., Ashani, Y., Khare, S. D., Oberdorfer, G., Leader, H., Baker, D. & Tawfik, D. S. Engineering V-type nerve agents detoxifying enzymes using computationally focused libraries. *ACS Chem. Biol.* **8,** 2394–2403 (2013).

20. Dawson, R. M. Review of oximes available for treatment of nerve agent poisoning. *J. Appl. Toxicol.* **14,** 317–331 (1994).

21. De Jong, L. P., van Dijk, C. & Benschop, H. P. Hydrolysis of the four stereoisomers of soman catalyzed by liver homogenate and plasma from rat, guinea pig and marmoset, and by human plasma. *Biochem. Pharmacol.* **37,** 2939–2948 (1988).

22. Désiré, B. & Saint-André, S. Interaction of soman with beta-cyclodextrin. *Fundam. Appl. Toxicol.* **7,** 646–657 (1986).

23. Désiré, B. & Saint-André, S. Inactivation of sarin and soman by cyclodextrins in vitro. *Experientia* **43,** 395–397 (1987).

24. Doctor, B. P. & Saxena, A. Bioscavengers for the protection of humans against organophosphate toxicity. *Chem. Biol. Interact.* **157-158,** 167–171 (2005).

25. Dodziuk, H. *Cyclodextrins and their complexes: chemistry, analytical methods, applications.* (ed Dodziuk, H.) (Wiley-VCH, Weinheim, 2006).

26. Eisenkraft, A., Gilburd, D., Kassirer, M. & Kreiss, Y. What can we learn on medical preparedness from the use of chemical agents against civilians in Syria? *Am. J. Emerg. Med.* **32,** 186 (2014).

27. Eto, M. *Organophosphorus pesticides: organic and biological chemistry.* (CRC Press, Boca Raton, FL, 1974).

28. Eyer, P. & Worek, F. in *Chemical warfare agents: toxicology and treatment.* (eds Marrs, T. C., Maynard, R. & Sidell, F.) 305–329 (John Wiley & Sons Ltd., Chichester, 2007).

29. Fukuto, T. R. Mechanism of action of organophosphorus and carbamate insecticides. *Environ. Health Perspect.* **87,** 245–254 (1990).

30. Goldsmith, M., Ashani, Y., Simo, Y., Ben-David, M., Leader, H., Silman, I., Sussman, J. L. & Tawfik, D. S. Evolved stereoselective hydrolases for broad-spectrum G-type nerve agent detoxification. *Chem. Biol.* **19,** 456–466 (2012).

31. Grob, D. The manifestations and treatment of poisoning due to nerve gas and other organic phosphate anticholinesterase compounds. *AMA Arch. Intern. Med.* **98,** 221–239 (1956).

32. Gunnell, D., Eddleston, M., Phillips, M. R. & Konradsen, F. The global distribution of fatal pesticide self-poisoning: systematic review. *BMC Public Health* **7,** 357 (2007).

33. Heath, A. J. W. & Meredith, T. in *Clinical and experimental toxicology of organophosphates and carbamates.* (eds Ballantyne, B. & Marrs, T. C.) 543–554 (Butterworth & Heinemann, Oxford, 1992).

34. Hedges, A. R. Industrial applications of cyclodextrins. *Chem. Rev.* **98,** 2035–2044 (1998).

35. Hennrich, N. & Cramer, F. Inclusion compounds. XVIII. The catalysis of the fission of pyrophosphates by cyclodextrin. A model reaction for the mechanism of enzymes. *J. Am. Chem. Soc.* **87,** 1121–1126 (1965).

36. Hobbiger, F. in *Cholinesterases and anticholinesterase agents.* (ed Koelle, G.) 921–988 (Springer, Berlin, 1963).

37. Holmstedt, B. Pharmacology of organophosphorus cholinesterase inhibitors. *Pharmacol. Rev.* **11,** 567–688 (1959).

38. Jeyaratnam, J. Acute pesticide poisoning: a major global health problem. *World Health Stat. Q* **43,** 139–144 (1990).

39. Johnson, M. K., Jacobson, D., Meredith, T., Eyer, P., Heath, A. J., Ligtenstein, D., Marrs, T. C., Szinicz, L., Vale, J. A. & Haines, J. Evaluation of antidotes for poisoning by organophosphorus pesticides. *Emerg. Med.* **12,** 22–37 (2000).

40. Kanagaraj, K., Affrose, A., Sivakolunthu, S. & Pitchumani, K. Highly selective fluorescent sensing of fenitrothion using per-6-amino-β-cyclodextrin:Eu(III) complex. *Biosens. Bioelectron.* **35,** 452–455 (2012).

41. Katz, F. S., Pecic, S., Tran, T. H., Trakht, I., Schneider, L., Zhu, Z., Ton-That, L., Luzac, M., Zlatanic, V., Damera, S., Macdonald, J., Landry, D. W., Tong, L. & Stojanovic, M. N. Discovery of new classes of compounds that reactivate acetylcholinesterase inhibited by organophosphates. *Chembiochem* **16,** 2205–2215 (2015).

42. Kirby, S. D., Norris, J. R., Richard Smith, J., Bahnson, B. J. & Cerasoli, D. M. Human paraoxonase double mutants hydrolyze V and G class organophosphorus nerve agents. *Chem. Biol. Interact.* **203,** 181–185 (2013).

43. Kovarik, Z., Maček Hrvat, N., Katalinić, M., Sit, R. K., Paradyse, A., Žunec, S., Musilek, K., Fokin, V. V., Taylor, P. & Radić, Z. Catalytic soman scavenging by the Y337A/F338A acetylcholinesterase mutant assisted with novel site-directed aldoximes. *Chem. Res. Toxicol.* **28,** 1036–1044 (2015).

44. Kovarik, Z., Radić, Z., Berman, H. A. & Taylor, P. Mutation of acetylcholinesterase to enhance oxime-assisted catalytic turnover of methylphosphonates. *Toxicology* **233,** 79–84 (2007).

45. Kronman, C., Cohen, O., Mazor, O., Ordentlich, A., Raveh, L., Velan, B. & Shafferman, A. Next generation OP-bioscavengers: a circulatory long-lived 4-PEG hypolysine mutant of F338A-HuAChE with optimal pharmacokinetics and pseudo-catalytic characteristics. *Chem. Biol. Interact.* **187,** 253–258 (2010).

46. Lamb, J. C., Steinberg, G. M. & Hackley, B. E. Isopropyl methylphosphonylated bisquaternary oximes; powerful inhibitors of cholinesterase. *Biochim. Biophys. Acta* **89,** 174–176 (1964).

47. Le Provost, R., Wille, T., Louise, L., Masurier, N., Müller, S., Reiter, G., Renard, P.-Y., Lafont, O., Worek, F. & Estour, F. Optimized strategies to synthesize β-cyclodextrin-oxime conjugates as a new generation of organophosphate scavengers. *Org. Biomol. Chem.* **9,** 3026–3032 (2011).

48. Lenz, D. E., Yeung, D., Smith, J. R., Sweeney, R. E., Lumley, L. A. & Cerasoli, D. M. Stoichiometric and catalytic scavengers as protection against nerve agent toxicity: a mini review. *Toxicology* **233,** 31–39 (2007).

49. Loftsson, T. & Brewster, M. E. Pharmaceutical applications of cyclodextrins: basic science and product development. *J. Pharm. Pharmacol.* **62,** 1607–1621 (2010).

50. Lorke, D. E., Kalasz, H., Petroianu, G. A. & Tekes, K. Entry of oximes into the brain: a review. *Curr. Med. Chem.* **15,** 743–753 (2008).

51. Lotti, M. in *Handbook of pesticide toxicology.* (ed Krieger, R.) 1043–85 (Academic Press, San Diego, 2001).

52. Luo, C., Ashani, Y. & Doctor, B. P. Acceleration of oxime-induced reactivation of organophosphate-inhibited fetal bovine serum acetylcholinesterase by monoquaternary and bisquaternary ligands. *Mol. Pharmacol.* **53,** 718–726 (1998).

53. Macilwain, C. Study proves Iraq used nerve gas. *Nature* **363,** 3 (1993).

54. Marrs, T. C. Organophosphate poisoning. *Pharmacol. Ther.* **58,** 51–66 (1993).

55. Marrs, T. C., Rice, P. & Vale, J. A. The role of oximes in the treatment of nerve agent poisoning in civilian casualties. *Toxicol. Rev.* **25,** 297–323 (2006).

56. Masson, P. Evolution of and perspectives on therapeutic approaches to nerve agent poisoning. *Toxicol. Lett.* **206,** 5–13 (2011).

57. Masson, P. & Lushchekina, S. V. Emergence of catalytic bioscavengers against organophosphorus agents. *Chem. Biol. Interact. (in press)* (2016).

58. Masurier, N., Estour, F., Froment, M.-T., Lefèvre, B., Debouzy, J.-C., Brasme, B., Masson, P. & Lafont, O. Synthesis of 2-substituted beta-cyclodextrin derivatives with a hydrolytic activity against the organophosphorylester paraoxon. *Eur. J. Med. Chem.* **40,** 615–623 (2005).

59. Maynard, R. L. & Beswick, F. W. in *Clinical and experimental toxicology of organophosphates and carbamates.* (eds Ballantyne, B. & Marrs, T. C.) 373–385 (Butterworth, Oxford, 1992).

60. Melzer, M., Chen, J. C.-H., Heidenreich, A., Gäb, J., Koller, M., Kehe, K. & Blum, M.-M. Reversed enantioselectivity of diisopropyl fluorophosphatase against organophosphorus nerve agents by rational design. *J. Am. Chem. Soc.* **131,** 17226–17232 (2009).

61. Michel, H. O., Hackley, B. E., Berkowitz, L., List, G., Hackley, E. B., Gillilan, W. & Pankau, M. Ageing and dealkylation of soman (pinacolylmethylphosphonofluoridate)-inactivated eel cholinesterase. *Arch. Biochem. Biophys.* **121,** 29–34 (1967).

62. Morimoto, F., Shimazu, T. & Yoshioka, T. Intoxication of VX in humans. *Am. J. Emerg. Med.* **17,** 493–494 (1999).

63. Mumford, H., Docx, C. J., Price, M. E., Green, A. C., Tattersall, J. E. H. & Armstrong, S. J. Human plasma-derived BuChE as a stoichiometric bioscavenger for treatment of nerve agent poisoning. *Chem. Biol. Interact.* **203,** 160–166 (2013).

64. Nachon, F., Brazzolotto, X., Trovaslet, M. & Masson, P. Progress in the development of enzyme-based nerve agent bioscavengers. *Chem. Biol. Interact.* **206,** 536–544 (2013).

65. Nagao, M., Takatori, T., Matsuda, Y., Nakajima, M., Iwase, H. & Iwadate, K. Definitive evidence for the acute sarin poisoning diagnosis in the Tokyo subway. *Toxicol. Appl. Pharmacol.* **144,** 198–203 (1997).

66. Newmark, C. J. The birth of nerve agent warfare: lessons from Syed Abbas Foroutan. *Neurology* **62,** 1590–1596 (2004).

67. Okuno, S., Sakurada, K., Ohta, H., Ikegaya, H., Kazui, Y., Akutsu, T., Takatori, T. & Iwadate, K. Blood-brain barrier penetration of novel pyridinealdoxime methiodide (PAM)-type oximes examined by brain microdialysis with LC-MS/MS. *Toxicol. Appl. Pharmacol.* **227,** 8–15 (2008).

68. Organisation for the Prohibition of Chemical Weapons. Convention on the prohibition of the development, production, stockpiling and use of chemical weapons and on their destruction. *The Hague* (1993).

69. Otero-Espinar, F. J., Luzardo-Alvarez, A. & Blanco-Méndez, J. Cyclodextrins: more than pharmaceutical excipients. *Mini-Rev. Med. Chem.* **10,** 715–725 (2010).

70. Radić, Z., Dale, T., Kovarik, Z., Berend, S., Garcia, E., Zhang, L., Amitai, G., Green, C., Radić, B., Duggan, B. M., Ajami, D., Rebek, J. & Taylor, P. Catalytic detoxification of nerve agent and pesticide organophosphates by butyrylcholinesterase assisted with non-pyridinium oximes. *Biochem. J.* **450,** 231–242 (2013).

71. Reiter, G., Mikler, J., Hill, I., Weatherby, K., Thiermann, H. & Worek, F. Chromatographic resolution, characterisation and quantification of VX enantiomers in hemolysed swine blood samples. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **873,** 86–94 (2008).

72. Rochu, D., Chabrière, E. & Masson, P. Human paraoxonase: a promising approach for pre-treatment and therapy of organophosphorus poisoning. *Toxicology* **233,** 47–59 (2007).

73. Saxena, A., Sun, W., Luo, C. & Doctor, B. P. Human serum butyrylcholinesterase: in vitro and in vivo stability, pharmacokinetics, and safety in mice. *Chem. Biol. Interact.* **157-158,** 199–203 (2005).

74. Saxena, A., Sun, W., Luo, C., Myers, T. M., Koplovitz, I., Lenz, D. E. & Doctor, B. P. Bioscavenger for protection from toxicity of organophosphorus compounds. *J. Mol. Neurosci.* **30,** 145–148 (2006).

75. Schrader, G. Organische Phosphor-Verbindungen als neuartige Insektizide. *Angew. Chemie* **62,** 471–473 (1950).

76. Sidell, F. R. Clinical effects of organophosphorus cholinesterase inhibitors. *J. Appl. Toxicol.* **14,** 111–113 (1994).

77. Szejtli, J. Introduction and general overview of cyclodextrin chemistry. *Chem. Rev.* **98,** 1743–1754 (1998).

78. Szinicz, L. History of chemical and biological warfare agents. *Toxicology* **214,** 167–181 (2005).

79. Talbot, B. G., Anderson, D. R., Harris, L. W., Yarbrough, L. W. & Lennox, W. J. A comparison of in vivo and in vitro rates of aging of soman-inhibited erythrocyte acetylcholinesterase in different animal species. *Drug Chem. Toxicol.* **11,** 289–305 (1988).

80. Tammelin, L. Dialkoxy-phosphorylthiocholines, alkoxy-methyl-phosphorylthiocholines and analogous choline esters. *Acta Chemica Scandinavia* **11,** 1340–1349 (1957).

81. Taylor, P., Radic, Z., Hosea, N. A., Camp, S., Marchot, P. & Berman, H. A. Structural bases for the specificity of cholinesterase catalysis and inhibition. *Toxicol. Lett.* **82-83,** 453–458 (1995).

82. Tenberken, O., Thiermann, H., Worek, F. & Reiter, G. Chromatographic preparation and kinetic analysis of interactions between tabun enantiomers and acetylcholinesterase. *Toxicol. Lett.* **195,** 142–146 (2010).

83. Trovaslet-Leroy, M., Musilova, L., Renault, F., Brazzolotto, X., Misik, J., Novotny, L., Froment, M.-T., Gillon, E., Loiodice, M., Verdier, L., Masson, P., Rochu, D., Jun, D. & Nachon, F. Organophosphate hydrolases as catalytic bioscavengers of organophosphorus nerve agents. *Toxicol. Lett.* **206,** 14–23 (2011).

84. Tsai, P.-C., Bigley, A., Li, Y., Ghanem, E., Cadieux, C. L., Kasten, S. A., Reeves, T. E., Cerasoli, D. M. & Raushel, F. M. Stereoselective hydrolysis of organophosphate nerve agents by the bacterial phosphotriesterase. *Biochemistry* **49,** 7978–7987 (2010).

85. United Nations. UN Mission to investigate allegations of the use of chemical weapons in the Syrian Arab Republic: report on allegations of the use of chemical weapons in Ghouta Area of Damascus on 21 August 2013. (2013).

86. Van Hooidonk, C. & Breebaart-Hansen, J. C. Stereospecific reaction of isopropyl methylphosphonofluoridate (sarin) with alphacyclodextrin. *Recueil* **89,** 289–299 (1970).

87. Volans, A. P. Sarin: guidelines on the management of victims of a nerve gas attack. *J. Accid. Emerg. Med.* **13,** 202–206 (1996).

88. Wille, T., Tenberken, O., Reiter, G., Müller, S., Le Provost, R., Lafont, O., Estour, F., Thiermann, H. & Worek, F. Detoxification of nerve agents by a substituted beta-cyclodextrin: application of a modified biological assay. *Toxicology* **265,** 96–100 (2009).

89. Wilson, I. B. Acetylcholinesterase. XI. Reversibility of tetraethyl pyrophosphate. *J. Biol. Chem.* **190,** 111–117 (1951).

90. Wolfe, A. D., Blick, D. W., Murphy, M. R., Miller, S. A., Gentry, M. K., Hartgraves, S. L. & Doctor, B. P. Use of cholinesterases as pretreatment drugs for the protection of rhesus monkeys against soman toxicity. *Toxicol. Appl. Pharmacol.* **117,** 189–193 (1992).

91. Worek, F., Seeger, T., Goldsmith, M., Ashani, Y., Leader, H., Sussman, J. S., Tawfik, D., Thiermann, H. & Wille, T. Efficacy of the rePON1 mutant IIG1 to prevent cyclosarin toxicity in vivo and to detoxify structurally different nerve agents in vitro. *Arch. Toxicol.* **88,** 1257–1266 (2014).

92. Worek, F., Seeger, T., Reiter, G., Goldsmith, M., Ashani, Y., Leader, H., Sussman, J. L., Aggarwal, N., Thiermann, H. & Tawfik, D. S. Post-exposure treatment of VX poisoned guinea pigs with the engineered phosphotriesterase mutant C23: a proof-of-concept study. *Toxicol. Lett.* **231,** 45–54 (2014).

93. Worek, F., Seeger, T., Zengerle, M., Kubik, S., Thiermann, H. & Wille, T. Effectiveness of a substituted β-cyclodextrin to prevent cyclosarin toxicity in vivo. *Toxicol. Lett.* **226,** 222–227 (2014).

94. Worek, F. & Thiermann, H. Reactivation of organophosphate-inhibited human acetylcholinesterase by isonitrosoacetone (MINA): a kinetic analysis. *Chem. Biol. Interact.* **194,** 91–96 (2011).

95. Worek, F. & Thiermann, H. The value of novel oximes for treatment of poisoning by organophosphorus compounds. *Pharmacol. Ther.* **139,** 249–259 (2013).

96. Worek, F., Thiermann, H., Szinicz, L. & Eyer, P. Kinetic analysis of interactions between human acetylcholinesterase, structurally different organophosphorus compounds and oximes. *Biochem. Pharmacol.* **68,** 2237–2248 (2004).

97. Worek, F., Thiermann, H. & Wille, T. Catalytic bioscavengers in nerve agent poisoning: A promising approach? *Toxicol. Lett.* **244,** 143–148 (2016).

98. Wright, P. G. An analysis of the central and peripheral components of respiratory failure produced by anticholinesterase poisoning in the rabbit. *J. Physiol. (Lond.)* **126,** 52–70 (1954).

99. Yeung, D. T., Smith, J. R., Sweeney, R. E., Lenz, D. E. & Cerasoli, D. M. Direct detection of stereospecific soman hydrolysis by wild-type human serum paraoxonase. *FEBS J.* **274,** 1183–1191 (2007).

100. Zengerle, M., Brandhuber, F., Schneider, C., Worek, F., Reiter, G. & Kubik, S. Highly efficient cyclosarin degradation mediated by a β-cyclodextrin derivative containing an oxime-derived substituent. *Beilstein J. Org. Chem.* **7,** 1543–1554 (2011).

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