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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).

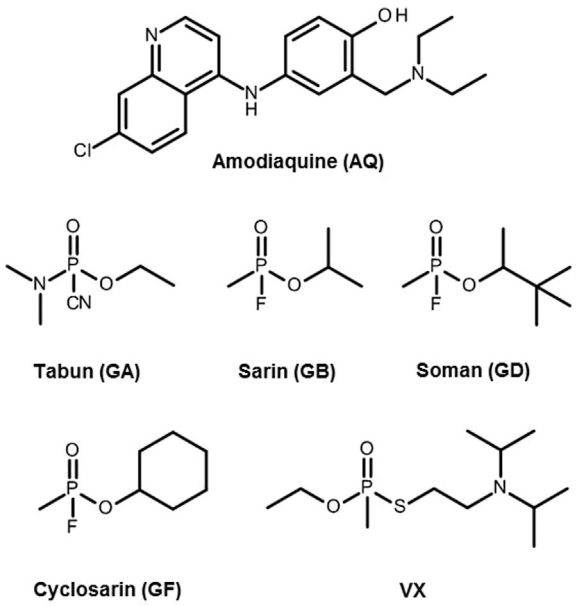

Fig. 1. Chemical structures of amodiaquine and organophosphorus compounds.

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