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REVIEW ARTICLE

SAR study to find optimal cholinesterase reactivator against organophosphorous nerve agents and pesticides

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Abstract Irreversible inhibition of acetylcholinesterase (AChE) by organophosphates leads to many failures in living organism and ultimately in death. Organophosphorus compounds developed as nerve agents such as tabun, sarin, soman, VX and others belong to the most toxic chemical warfare agents and are one of the biggest threats to the modern civilization. Moreover, misuse of nerve agents together with organophosphorus pesticides (e.g. malathion, paraoxon, chlorpyrifos, etc.) which are annually implicated in millions of intoxications and hundreds of thousand deaths reminds us of insufficient protection against these compounds. Basic treatments for these intoxications are based on immediate administration of atropine and acetylcholinesterase reactivators which are currently represented by mono- or bis-pyridinium aldoximes. However, these antidotes are not sufficient to ensure 100 % treatment efficacy even they are administered immediately after intoxication, and in general, they possess several drawbacks. Herein, we have reviewed new efforts leading to the development of novel reactivators and proposition of new promising strategies to design novel and effective antidotes. Structure–activity relationships and biological activities of recently proposed acetylcholinesterase reactivators are discussed and summarized. Among further modifications of known oximes, the main attention has been paid to dual binding site ligands of AChE as the current mainstream strategy. We have also discussed new chemical entities as potential replacement of oxime functional group.

Keywords Acetylcholinesterase · Organophosphate · Pyridinium oximes · Reactivation · Nerve agents · Uncharged reactivator

Introduction

Chemical warfare agents (CWAs) are one of the big threats in the modern civilization. This fact is supported by the expanded power of terrorism which endangers any country worldwide. The most toxic CWAs are nerve agents, chemically classified as organophosphorus compounds (OPs) (denoted for both organophosphates and organophosphonates) (Watson et al. 2015). Nerve agents can be divided into two major groups. The first group labelled as "G-agents" containing tabun (GA), sarin (GB), soman (GD) and cyclosarin (GF) contains more volatile substances, therefore representing a vapour and liquid threat, however, with rather lower persistency in open terrain. The second group of "V-agents" including VX (originates from Great Britain), CVX (Chinese isomer) and R-VX (VR; Russian isomer) are relatively non-volatile with high persistency in the environment and increased toxicity (Fig. 1) (Kuca and Pohanka 2010; Mercey et al. 2012b).

Misuse of aggresive nerve agents was expressively demonstrated during the Gulf War (MacIlwain 1993; Worek

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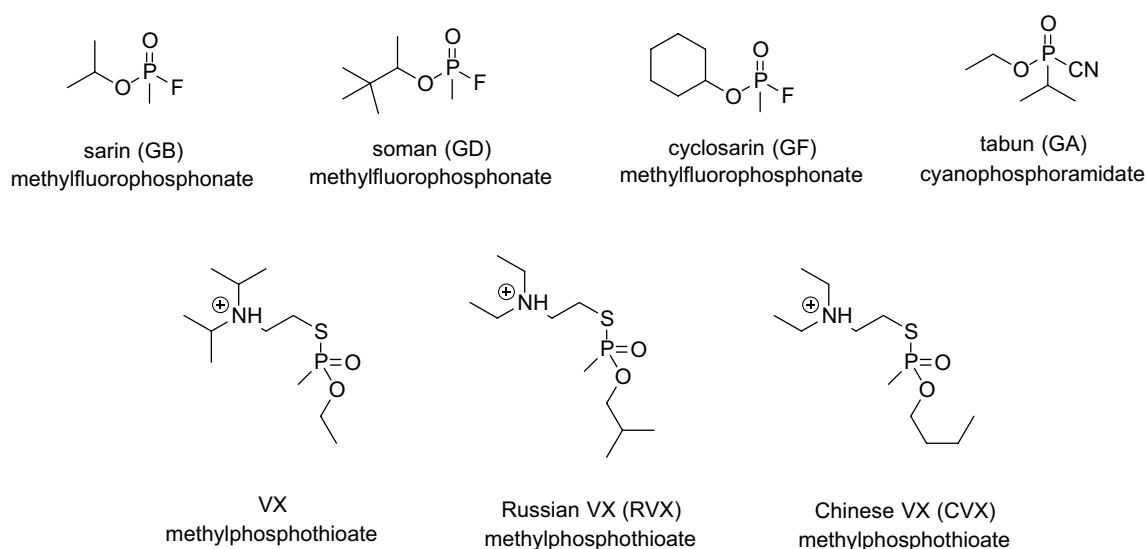
Fig. 1 Chemical structures of chemical warfare nerve agents

and Thiermann 2013), terrorist attacks in Matsumoto city (1994), in the Tokyo subway (1995) with sarin and in Osaka (1994) with VX-agent by religious cult Aum Shinrikyo (Agarwal et al. 2004). Furthermore, recent sarin attacks in the Syria recall the attention to counteracting the CWAs misuse and the importance of the further investigation in finding effective nerve agent antidotes (Pita and Domingo 2014; Winter et al. 2015). Besides CWAs, less toxic class of organophosphorus compounds may represent serious public health issue as well. Organophosphorus-based pesticides are annually implicated in more than 3,000,000 acute intoxications which lead in 200,000 fatalities (Eddleston et al. 2008; Mercey et al. 2012b).

The toxic effect of OPs (nerve agents and pesticides) is based on irreversible inhibition of enzyme acetylcholinesterase (AChE, E.C. 3.1.1.7) (Millett 2006). AChE is an inextricable component of cholinergic synapses and neuromuscular junctions. Its main biological function consists in rapid hydrolysis of the positively charged neurotransmitter acetylcholine (ACh) (Tougu 2001). Inhibition of AChE with OPs proceeds through the formation of covalent P–O bond in the catalytic active site (CAS) of AChE, which leads to accumulation of ACh in synapses and subsequent overstimulation of cholinergic receptors resulting in cholinergic crisis, convulsive seizures, respiratory distress and, ultimately, death (Marrs 1993; Wei et al. 2014). The current treatment of OP poisoning consists of administration of the combination of causal and symptomatic drugs. The symptomatic antidotes include anticonvulsives (e.g. diazepam) and anticholinergic drugs where atropine plays a pivotal role. AChE reactivators (oximes) are causal drugs which are able to recover AChE function. The mechanism of reactivation is believed to include nucleophilic attack of

phosphorus atom of inhibited AChE, resulting in removal of OP bond and recovery of enzyme's catalytic activity (Kassa 2002; Ruark et al. 2013; Soukup et al. 2013a). However, also non-reactivating properties of oximes are often discussed. Namely, the direct interactions with the cholinergic receptors, high affinity choline reuptake or other parts of cholinergic system are mentioned in order to explain a life-saving effect after the ageing (Tattersall 1993; van Helden et al. 1996; Soukup et al. 2011, 2012; Sepsova et al. 2014). Pralidoxime (2-PAM), methoxime (MMB-4), trimedoxime (TMB-4), obidoxime (LüH-6) and asoxime (HI-6) are the best studied and commercially available oxime reactivators (Fig. 2) (Wilson and Ginsburg 1955; Poziomek et al. 1958; Hobbiger et al. 1958; Luettringhaus and Hagedorn 1964; Hagedorn et al. 1969; Bajgar 2012; Jokanović 2012).

AChE action and inhibition

AChE enzyme belongs to the α/β hydrolase family including cholinesterases, carboxylesterases and lipases. Its main function is to terminate nerve impulses via hydrolysis of ACh in the synapses (Tougu 2001; Lushington et al. 2006). AChE is considered to be an evolutionary perfect enzyme as it is responsible for substrate association, chemical transformation and product dissociation at similar rates with high efficiency degradation of 2.5×10^{-4} ACh molecules per second (Scheme 1) (Quinn 1987; Ordentlich et al. 1993; Shaikh et al. 2014; Rosenberry 2006). Another member of the cholinesterase family is presented by butyrylcholinesterase (BChE; EC 3.1.1.8) which is also able to hydrolyse ACh and bulkier substrates (e.g. butyrylcholine); however, its physiological role is of minor importance compared to AChE. BChE takes part in detoxification and metabolism
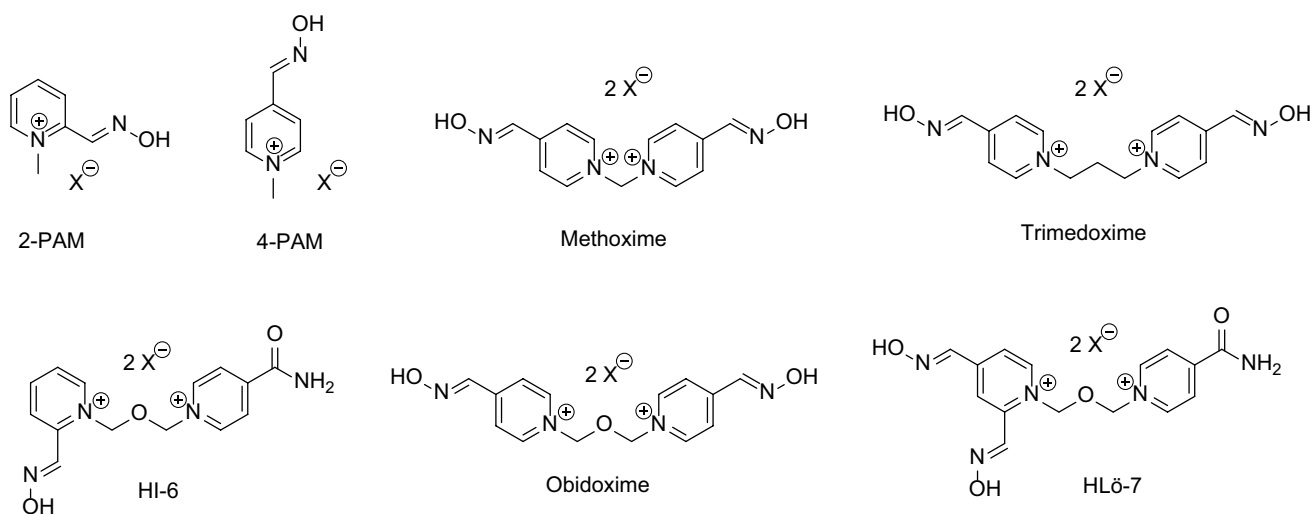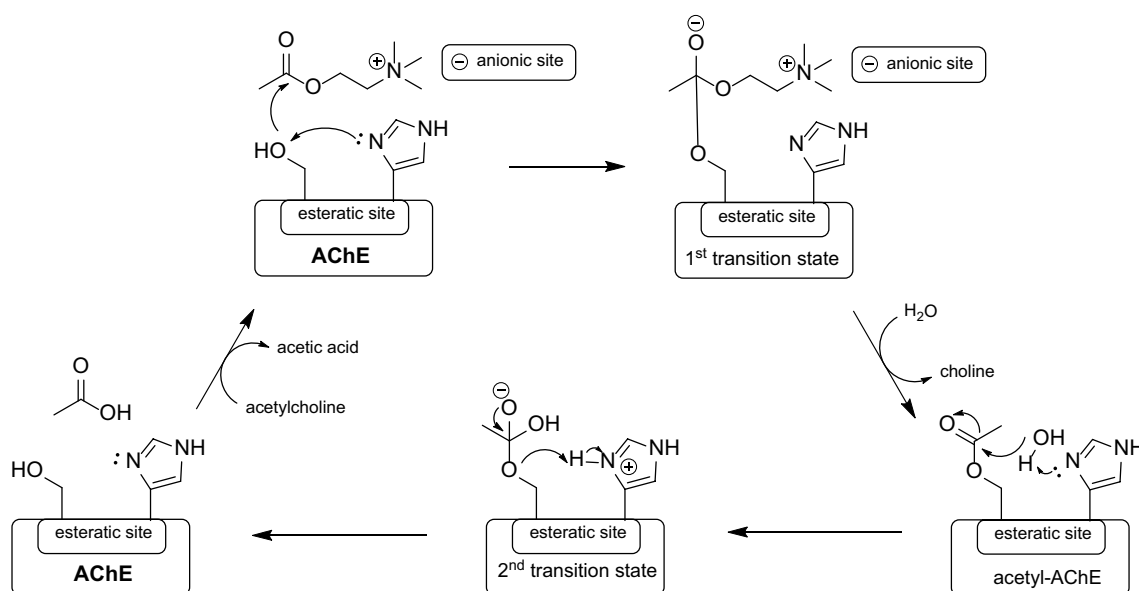
Fig. 2 Oxime reactivators used as antidotes for OP-inhibited AChE

**Scheme 1** Mechanism of ACh hydrolysis by AChE

processes of ester compounds (e.g. aspirin, physostigmine and cocaine) even though the real physiological role remains unclear (Lockridge 2015; Maurice et al. 2016). Recently, BChE found its application as a biomarker of OP-exposure or as a OP-scavenger (Broomfield et al. 1991; Fidder et al. 2002; Horn et al. 2015; Lockridge 2015).

Structure of AChE was firstly detailed in 1991 by J. Sussman on *Torpedo californica* acetylcholinesterase (*Tc*AChE). *Tc*AChE is composed of 537 amino acids with two main sites connected with narrow gorge: catalytic active site (CAS) and peripheral anionic site (PAS) (Fig. 3) (Sussman et al. 1991). Many studies have been subsequently focused on proper structure and mechanism of the

function (Johnson and Moore 2006; Berg et al. 2011; Singh et al. 2013; Bajda et al. 2013).

Several ways to inhibit AChE activity have been proposed. Organophosphates and carbamates bind competitively and directly to the active site serine (Johnson and Moore 2006). The mechanism of OP action is similar to the initial step of ACh hydrolysis. OPs penetrate through the gorge of AChE to the catalytic active site. Nucleophilic serine attacks the phosphorus atom, forming a bipyramidal transition state, which release halide ion and the formation of phosphylated serine (Scheme 2). In the second step, histidine residue cannot fulfil its role of water activation, and therefore, spontaneous hydrolysis is extremely slow
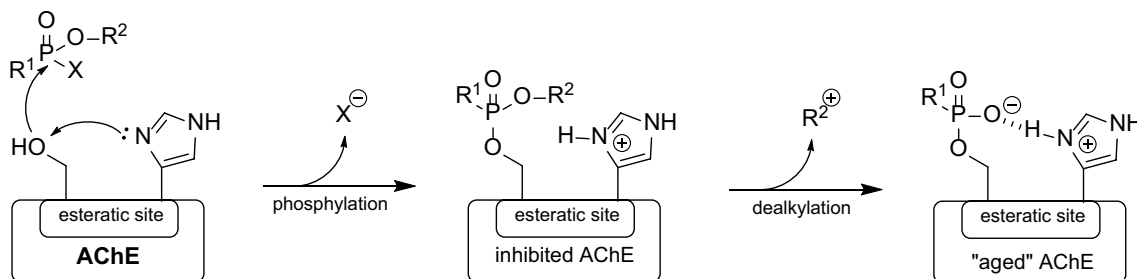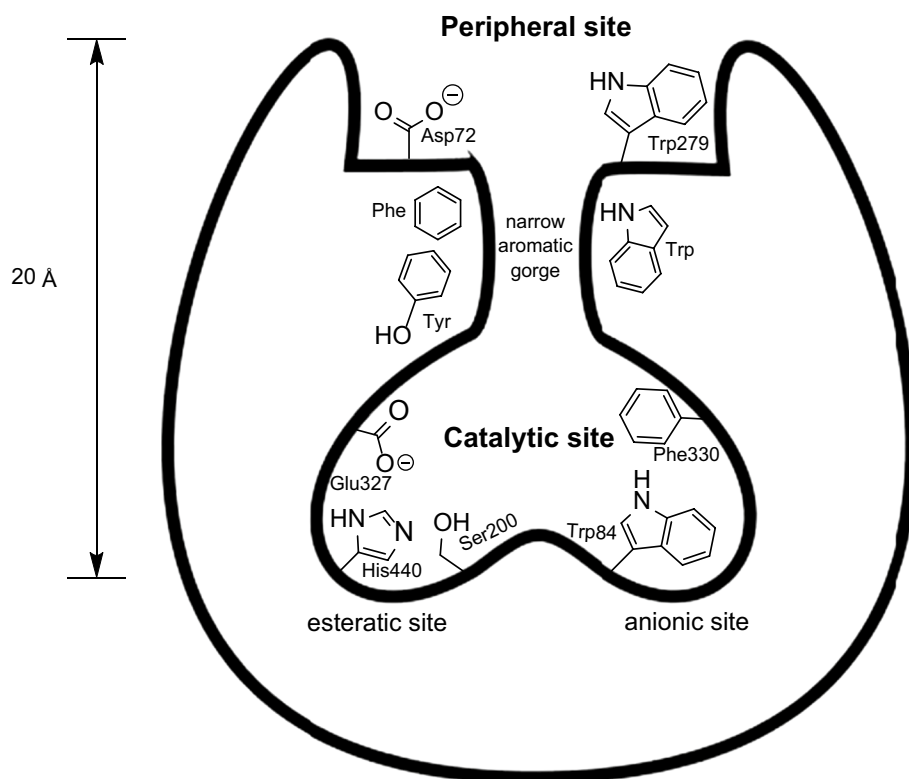
Scheme 2 Inhibition of AChE by organophosphorus nerve agents and "ageing" process

varying from hours to days (Mercey et al. 2012b). This failure in the hydrolysis is explained by formation of unsuitable conformation (e.g. VX and tabun conjugates) (Millard et al. 1999; Carletti et al. 2010) or shielding from the water molecule of histidine (e.g. soman conjugate) (Sanson et al. 2009). Besides spontaneous hydrolysis of OP conjugates, time-dependent intramolecular reaction appears. This process involves dealkylation of alkoxy substituent from the phosphorus atom yielding to the phosphonate adducts denoted as "aged" form of conjugate (Masson et al. 2010). The "aged" conjugate forms a salt bridge with the protonated catalytic histidine resulting in the strong stabilization (Segall et al. 1993; Carletti et al. 2010). Such "aged" AChE is resistant to the hydrolysis and reactivation by oxime antidotes. The ageing half-time depends on the type of OP, differing from approximately 2–4 min for soman, 5 h for

sarin, 46 h for tabun and 48 h for VX (Worek et al. 2004; Mercey et al. 2012b).

Reactivation and drawbacks of common reactivators

More than 50 years ago, Irwin Wilson and his colleagues demonstrated that only strong nucleophiles such as oximes are able to reactivate OP-AChE conjugates and release free enzyme. For successful enzyme reactivation, it is important to accomplish three factors including strong nucleophilicity, proper orientation towards phosphate-enzyme adduct and to prevent process of "ageing" (Wilson and Ginsburg 1955; Wilson 1959; Froede and Wilson 1970; Wong et al. 2000). Dissociation constant (pK_a) is another important factor for the removal of phosphyl moiety due to the fact that reactivation is provided by dissociated oximate anion.
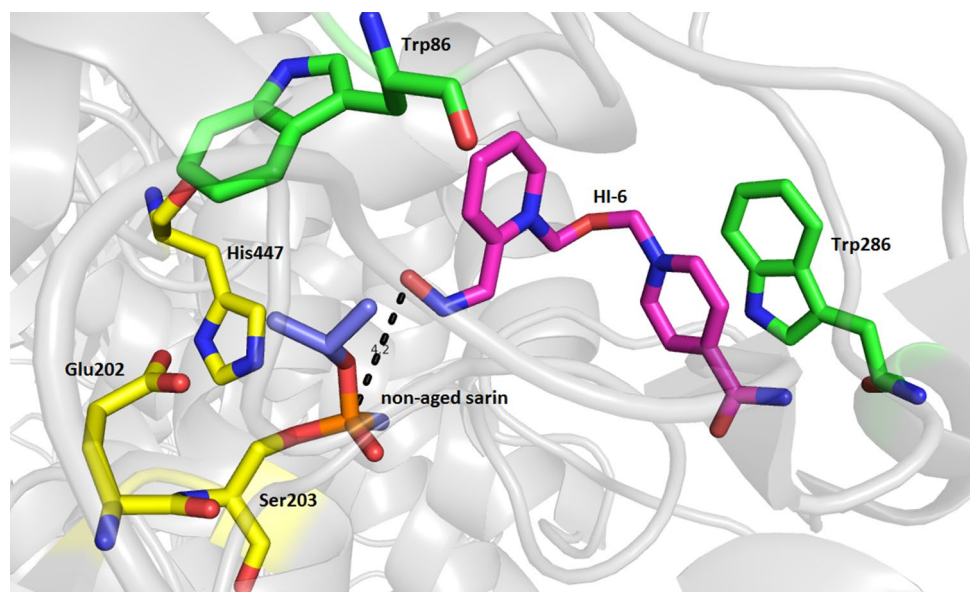
Fig. 4 Ternary complex of non-aged sarin-inhibited *Mus musculus* AChE with HI-6. Catalytic triad residues are rendered in *yellow carbon atoms*, sarin nerve agent in *blue carbon atoms* and HI-6 in *purple carbon atoms*. Tryptophans (*green carbon atoms*) delineate PAS (Trp286) and CAS (Trp86) of AChE. The rest of the enzyme is displayed as *grey cartoon*. Oximate group of HI-6 is oriented towards phosphorus atom of sarin in the distance of 4.2 Å suggesting proper conformation for reactivation of the enzyme (Allgardsson et al. 2016). Figure was created with PyMol 1.5.0.4 (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC, Mannheim, Germany)

Moreover, proper pK_a is the factor limiting penetration through the biological membranes (Pajouhesh and Lenz 2005; Musil et al. 2016). Therefore, proper acidity (pK_a) of the oxime group is very important (pK_a values for standard reactivators range from 7.04 for HLö7 to 8.2 for trimedoxime) (Wilson and Ginsburg 1959; Hagedorn et al. 1972; Čakar et al. 1999). De Jong revealed that the attachment of oxime moiety to pyridinium ring forms another crucial feature necessary for successful enzyme reactivation. He also suggested that the oxime group in position 2 is important for the reactivation of soman-inhibited AChE, while the oxime group in position 4 is superior for the reactivation of tabun-inhibited AChE (de Jong et al. 1989). Another study demonstrated that the 4-positioned oximes are superior compared to 2-positioned oximes in the case of pesticides and phosphoramidates with substituted amido groups (tabun) (Worek et al. 2004). It was also shown that in the case of soman-inhibited AChE, structure–activity relationships (SARs) of oximes cannot be applied for all the OPs (Worek et al. 2004). Furthermore, another investigation pointed that substituted amide groups reduce electrophilicity of the phosphorus atom, thus preventing nucleophilic attack by oxime function which is supported with additional steric hindrance of tabun alkyl chain (Worek et al. 2004). Therefore, tabun-inhibited AChE is supposed to be one of the most resistant OP regarding reactivation by oximes (Heilbronn 1963). Several other studies with molecular modelling, site-directed mutagenesis and X-ray crystallography were focused to uncover the detailed mechanism for reactivation of AChE (Fig. 4) (Ashani et al. 1995; Wong et al. 2000; Luo et al. 2003; Kovarik et al. 2004; Ekström et al. 2009; Artursson et al. 2009; Dolezal et al. 2015). Finally, the researchers' effort indicated that the structure of oxime must correspond to the specific structure of OP. The main factors like the orientation of phosphyl moiety in the active centre, spatial restriction and steric limitation affect the oxime entry and its reactivation potency. Therefore, a wide variety of reactivators can be used for different types of OP (Worek et al. 2004).

The reactivation of phosphylated AChE proceeds through the two-step reaction. Firstly, the oximate anion approaches to the P–O covalent bond between OP and the active site serine and forms a fully reversible Michaelis-type oxime-phosphyl-AChE-conjugate. Subsequently, the displacement of the phosphyl residue follows from the pentacoordinate transition state, releasing the free enzyme and the formation of a phosphylated oxime (Scheme 3) (Ekström et al. 2009; Artursson et al. 2009; Sanson et al. 2009; Worek and Thiermann 2013). Unfortunately, it has been demonstrated that the formed phosphyloxime is still a highly potent inhibitor capable to sufficiently re-inhibit AChE (Lamb et al. 1964; Luo et al. 1999). The latter mentioned phenomena can occur especially in the case of 3- or 4-positioned oxime group on the pyridinium ring. On the
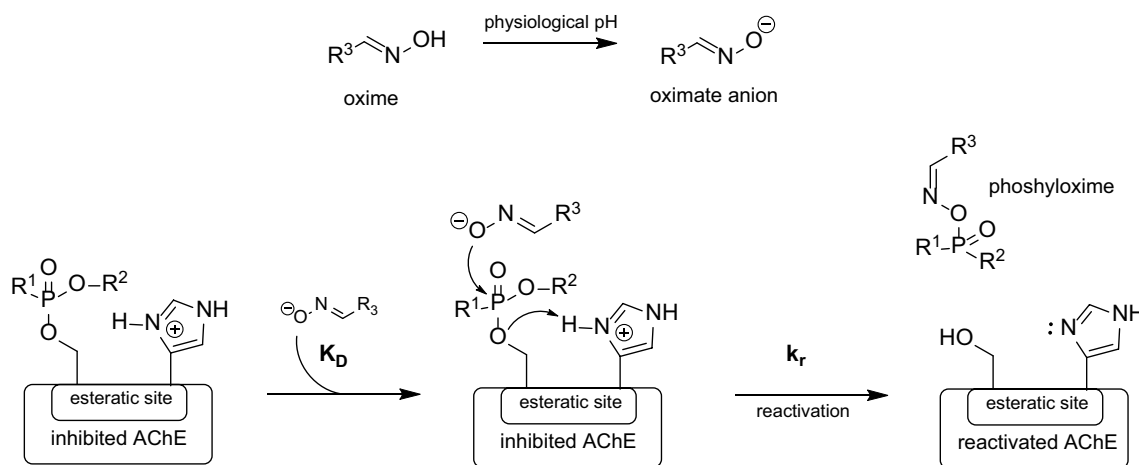
Scheme 3 Reactivation of OP-inhibited AChE with oximate anion leading into reactivated free enzyme and phosphyloxime (K_D-dissociation constant of the reactivator/phosphyl-AChE complex; k_r-reactivation rate constant)

contrary, 2-positioned oxime is less liable to such AChE re-inhibition (Worek et al. 2000; Ashani et al. 2003; Kiderlen et al. 2005; Stenzel et al. 2007).

All commercially available reactivators are charged oximes with one or two pyridinium rings connected through the different linkers (Fig. 2). First efficient oxime, pralidoxime (2-PAM), was described in the USA in 1955, and its characteristic structural feature consists of mono-pyridinium ring and oxime group in position 2 (Wilson and Ginsburg 1955). It is effective in the reactivation of sarin- and VX-inhibited AChE, but hardly reactivates soman- or tabun-inhibited-AChE (Koplovitz and Stewart 1994). First, bis-pyridinium oxime introduced to the market was methoxime, and it showed improved efficacy against diisopropylfluorophosphate- (DFP), tabun-, sarin- and VX-inhibited AChE compared to the 2-PAM and 4-PAM (Poziomek et al. 1958; Wilson and Ginsburg 1959). However, it is still inefficient in soman poisoning (Inns and Leadbeater 1983). Trimedoxime together with obidoxime and HLö-7 is considered as the most efficient in the case of tabun intoxication (Cabal et al. 2004; Carletti et al. 2009). On the other hand, they are less efficient for VX-inhibition (Segall et al. 1993). Asoxime (HI-6) is one of the most potent agents for treatment of soman intoxication; however, it is still inefficient against tabun (Worek et al. 2007a). Obidoxime is considered to be the best reactivator of pesticides (Worek et al. 2007b). However, it is important to keep in mind that none of these compounds represents universal reactivator even that the bis-pyridinium analogues are more efficient than mono-pyridinium ones (Segall et al. 1993; Jokanović 2012).

Therefore, a therapeutic value of oxime reactivators is still insufficient due to three main drawbacks. (1) Firstly, there is no broad-spectrum reactivator able to efficiently restore AChE activity after intoxication by different types of OPs (Korabecny et al. 2014). (2) Secondly, all marketed oxime reactivators are permanently charged and their permeation through the blood–brain barrier (BBB) is rather poor. Thus, they are readily unable to reactivate OP-inhibited AChE in the brain. Specifically, 2-PAM crosses BBB in approximately 10 % of the intravenously given dose. Bis-pyridinium oximes permeate through BBB even at lower level, approximately 1–3 % after intravenous administration (Sakurada et al. 2003; Bajgar et al. 2007a; Karasova et al. 2013). (3) Third drawback concerns about the "aged" AChE. Among all known oxime reactivators, there is none which could deal with these phenomena and be able to reactivate "aged" enzyme (Mercey et al. 2012b; Korabecny et al. 2014). However, life-saving efficacy was observed after the asoxime treatment of aged AChE probably due to non-reactivating mechanisms of this compound (Hamilton and Lundy 1989).

All these drawbacks still force the researchers to develop novel oxime antidotes which could serve as universal antidotes in all cases of OP intoxications. In the current study, we are discussing novel trends in reactivators' discovery with particular emphasis on AChE dual binding site reactivator strategy. Moreover, our contribution deals with different CAS and PAS ligands connected through the variety of linkers.

Catalytic active site ligands

Nowadays, the most effective AChE reactivators represent mono- or bis-quaternary pyridinium aldoximes, and they are still the leading moieties in the modern design and development of new reactivators. Formerly, the bivalent metal ions were probably able to potentiate the activity

Fig. 5 Pd(II)-obidoxime complexes (Nedzhib et al. 2014)

$[PdHL]^{3+}$

0 hours

$[Pd_2L]^{4+}$

24 hours
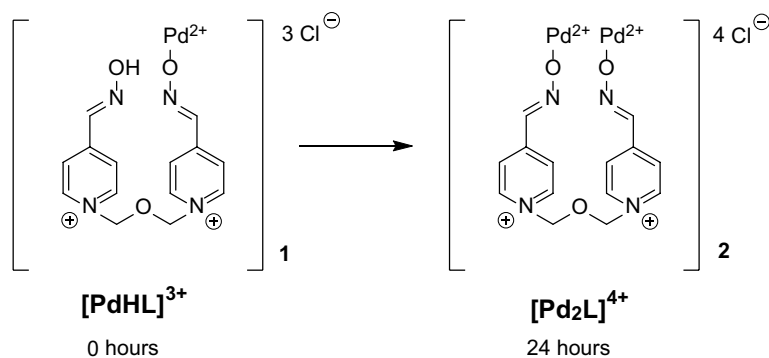
Fig. 6 Bis-quaternary 2-(hydroxyimino)-*N*-(pyridine-3-yl)acetamide derivatives (Karade et al. 2014)

n = 2 = **3**
3 = **4**
4 = **5**
5 = **6**
6 = **7**
7 = **8**

9 = 1,2-Phenylene
10 = 1,3-Phenylene
11 = 1,4-Phenylene
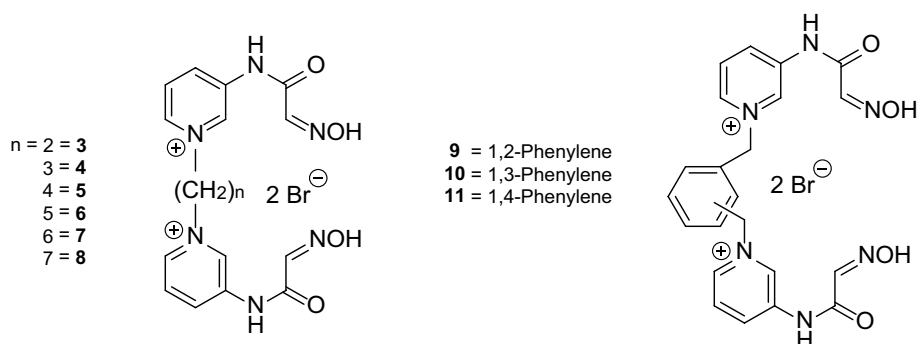
of AChE reactivator pralidoxime by formation of the corresponding complex species (Cier et al. 1969). However, one of the most versatile tools which is still frequently used as a reactivator in the therapy of organophosphorus nerve agents (OPNAs) intoxication is obidoxime (Kuca et al. 2009). Therefore, the attention was turned to preparation of Pd(II) complexes with obidoxime (Nedzhib et al. 2014). The authors of the study anticipated that the coordination form of obidoxime should be slowly decomposed compared to obidoxime itself thus subsequently increasing the amount of active compound in the organism (Cier et al. 1969; Nedzhib et al. 2014). At pH 7.4, the complex $[PdHL]^{3+}$ between Pd(II) ions and obidoxime was immediately and readily formed. These results are in agreement with the reported data of the previous research (Karljiković-Rajić et al. 1987; Nedzhib et al. 2014). The study revealed that obidoxime acts in a monodentate coordination with its oximate moiety. Within 24 h at pH 7.4, second Pd(II) ion is coordinated forming final product $[Pd_2L]^{4+}$. The final molar ratio metal-to-ligand is 2:1, and thus the formation of $[PdHL]^{3+}$ (0 h) and $[Pd_2L]^{4+}$ (24 h) depends on time and does not significantly change with the excess of the metal ion (Fig. 5). The reactivation ability of the new obidoxime complexes was established against paraoxon-inhibited rat brain AChE in in vitro conditions compared to the non-coordinated obidoxime indicating that obidoxime complexes with Pd(II) possess only very low reactivation potency (Nedzhib et al. 2014).

Disclosure of novel bis-pyridinium AChE reactivators revealed compounds containing 2-(hydroxyamino)-*N*-(pyridin-3-yl)acetamide (Fig. 6) (Karade et al. 2014). The presence of the electron-withdrawing functional group such as amide in conjugation to the oxime group can improve its dissociation into the active oximate anion. The attachment of the reactivation moiety in the 3-position of the side chain conferred p*K*a values ranging between 7.95 and 8.25 which is similar to that of the 2-PAM and obidoxime (Kuca et al. 2006; Musil et al. 2016). Bis-quaternary 2-(hydroxyimino)-*N*-(pyridine-3-yl)acetamide derivatives (**3**–**11**, Fig. 6) were assayed in vitro for their reactivation potency against sarin and VX using erythrocyte ghost *h*AChE. Analogues **3**–**11** were compared with 2-PAM and obidoxime. From this series, oximes **3**, **6**, **7** and **11** displayed potential for the reactivation of OPNAs inhibited *h*AChE comparable with pralidoxime (Karade et al. 2014).

Since the permanently charged oximes can hardly cross BBB, studies in the past few years switched more to the development of new uncharged reactivators. Several working groups have turned their efforts to novel centrally active reactivators exploiting different catalytic active site ligands as reactivation scaffolds.

One of the possibilities of uncharged reactivator was presented by Saint-André et al. (2011). The library of α-nucleophiles was screened in order to find a new highly efficient scaffold to reactivate VX-inhibited recombinant *h*AChE (Saint-André et al. 2011). The study
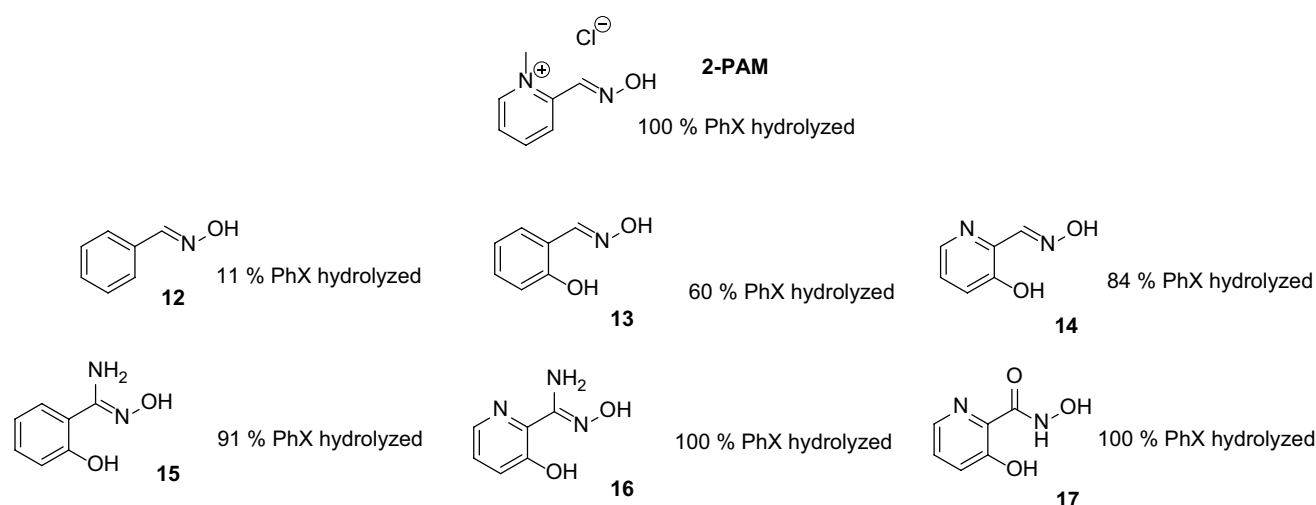
Fig. 7 Highlighted α-nucleophiles represented by arylaldoximes **12**, **13**, pyridylaldoxime **14**, arylamidoxime **15**, pyridylamidoxime **16** and pyridylhydroxamic acid **17** and their comparison to 2-PAM on PhX-hAChE (Saint-André et al. 2011)

on the hydrolysis ability of novel α-nucleophiles was performed on less toxic phenylphosphonothioate (PhX), derivative of VX with similar hydrolysis profile, due to the safety reasons (Vayron et al. 2000; Saint-André et al. 2011). As the reference compound, pralidoxime was selected, which hydrolysed PhX-hAChE inhibited complex quantitatively (100 %) under the studied conditions. Initially, subset consisting of aryl and pyridinealdoximes was used. Structure–activity relationship disclosed that *ortho*-monosubstituted aryls bearing different electron-donating and electron-withdrawing groups did not have significant effect on the hydrolytic activity. Interestingly, hydroxyl or carboxyl group in *ortho*-position showed enhanced hydrolytic activity. This might be explained by the intramolecular hydrogen bonding between hydroxyl group and nitrogen atom of oxime, decreasing the pKa value of the α-nucleophile (e.g. **12** pK_a = 10.9 versus **13** pK_a = 9.3, Fig. 7) (Blatt and Russell 1936; Hayashi et al. 1983; Saint-André et al. 2011). Following the initial findings, the hydroxyl group-containing α-nucleophiles were tested proposing *ortho*-hydroxybenzaldoxime with some electron-donating or electron-withdrawing moieties. Same effect was observed for pyridinealdoxime bearing hydroxyl group in *ortho*-position **14**. Moreover, the pyridine ring contributed to the decrease of pK_a value (pK_a = 8.2). Consequently, aryl and pyridinamidoximes (Louise-Leriche et al. 2010) were evaluated as novel promising reactivators with the *ortho*-hydroxyl group. Moreover, it was found that pKa values of α-hydroxybenzamidoximes **15** (pK_a = 8.88) and especially α-hydroxylpyridylamidoxime **16** (pK_a = 7.96) lies in the optimal pK_a range for improved reactivation ability compared to α-hydroxy aldoximes. Finally, the

reactivation properties of hydroxamic acid were also investigated. *Ortho*-hydroxyl substituted hydroxamic acid analogue **17** does not significantly increase hydrolysis of PhX-hAChE inhibited complex. On the other hand, pyridyl hydroxamic acids containing *ortho*-hydroxyl displayed improved hydrolytic activity. The most active α-nucleophiles under the survey were studied for their ability to reactivate hAChE inhibited by VX-agent in comparison with 2-PAM using spectrophotometric Ellman's protocol. Oximes **14** and **16** have showed high reactivation capability, and especially k_r was higher than for the reference compound 2-PAM. However, these nucleophiles exhibited much lower affinity to the inhibited enzyme with high dissociation constants (K_D), and therefore, much higher concentrations of these compounds were required to reactivate the enzyme (Saint-André et al. 2011).

Ongoing study by prof. Renard and co-workers followed exploitation of favourable properties and structural features of CAS ligands, especially fragments **14** and **16**, in combination with various linkers and PAS ligands as potential reactivators of OP-inhibited AChE. As a result, the first uncharged reactivators were reported (Mercey et al. 2011, 2012a; Renou et al. 2013; Kliachyna et al. 2014) using **14** or **16** fragment as reactivation moiety in attachment to PAS ligands such as phenyl-tetrahydroisoquinoline (PIQ) or tetrahydroacridine (THA). THA, originally developed as Alzheimer's disease drug acting as acetylcholinesterase inhibitor, was used as known CAS and PAS binder (Soukup et al. 2013b; Zemek et al. 2014).

Some fluorine-containing pyridine aldoximes were originally prepared in the period 1963–1966 at the University of Manchester, Institute of Science and Technology
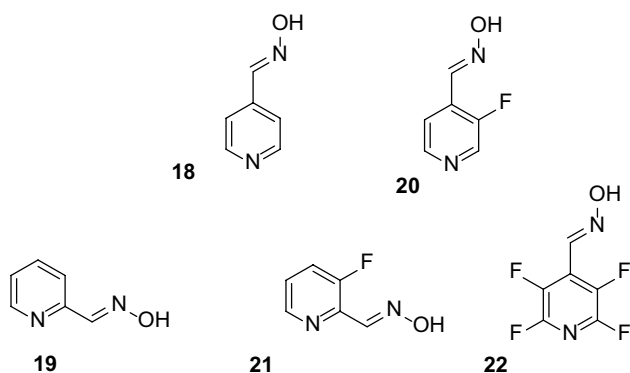
Fig. 8 Uncharged fluorinated and non-fluorinated pyridine aldoximes (Timperley et al. 2011)

Fig. 9 Structure of the most potent uncharged reactivator 23 (Sit et al. 2011)

Fig. 10 General structural pattern of imidazole aldoxime active for reactivation of OP-inhibited hBChE (Sit et al. 2011)

Fig. 11 Improved analogue of 23, azepine N-substituted 2-hydroxy-iminoacetamide 25 (Radić et al. 2012)

(UMIST) (I.M. Young 1966; Timperley et al. 2011). Then, between the years 2009 and 2010, fluorinated pyridinium aldoximes received particular attention again as potential antidotes against OP-inhibited AChE (Jeong et al. 2009a, b; Kassa et al. 2010). However, the reactivation experimental details were firstly discussed in current study (Timperley et al. 2011). The main idea of using the fluorinated pyridine aldoximes was to lower the pK_a to optimal values, and thus, the oximes should be able to form oximate anion under the physiological conditions (pH 7.4, 37 °C) (Kuca et al. 2006; Musil et al. 2016). Uncharged pyridine aldoximes 18–19 have pK_a > 10. Pyridine aldoximes with one fluorine atom (20–21) still remain far from the optimal values, whereas (E)-2,3,5,6-tetrafluoroisonicotinaldehyde oxime (22) has pK_a close to about 9.1 (Fig. 8). The study also suggested that introduction of some powerful electron-withdrawing groups (e.g. CF_3, CF_2H, SF_5, NO_2, CN, CF_3SO_2 and CF_3CH_2) might lead to decrease of the pK_a into the optimal values (Timperley et al. 2011).

Replacement of the pyridine ring by different heteroaryls such as imidazole was also explored in order to find novel AChE reactivators. Quaternary imidazolium aldoximes were investigated as potential reactivators in the early 1990s (Goff et al. 1991; Koolpe et al. 1991; Mesic et al. 1992). Very recently, non-quaternary imidazolium aldoximes were reported as reactivators of OP-inhibited AChE by Sit, Radic and Kovarik co-workers (Sit et al. 2011; Radić et al. 2012; Kovarik et al. 2013; Sit et al. 2014). Firstly, library of 134 novel structurally diverse uncharged oximes containing primary imidazole aldoximes and N-substituted 2-hydroxyiminoacetamides was screened (Sit et al. 2011). Their reactivation ability was assayed against the paraoxon, and sarin, cyclosarin and VX-inhibited hAChE compared to the 2-PAM. 56 acetamide oximes were found to be approximately equally efficient as 41 imidazole derivatives, whereas none of the remaining structurally variable oximes showed sufficient reactivation potency. Finally,
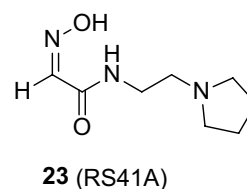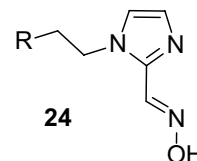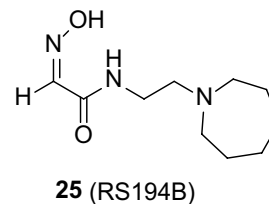
N-substituted 2-hydroxyiminoacetamides provided higher efficacy, especially with the five or six-methylene tether between oxime and PAS ligand, highlighting the structure of compound 23 (RS41A; Fig. 9) (Sit et al. 2011).

The library also included 64 novel oximes with triazole moiety acting as PAS ligand. However, reactivation potency of these triazole analogues did not reached reactivation ability of other compounds. The investigation also included reactivation of OP-inhibited hBChE. In relation to 2-PAM, reactivation potency of the subset of 100 uncharged oximes was explored. No correlation was found between reactivation of OP-inhibited hBChE and OP-inhibited hAChE most probably due the structure diversity of these enzymes. However, the general structural pattern for the most active reactivators for OP-inhibited hBChE turned out to be uncharged imidazole oxime with the alkyl chain 24 (Fig. 10) (Sit et al. 2011).

This study was further extended dealing with modified structures based on 23 (RS41A) (Radić et al. 2012). As a consequence, this study disclosed new lead azepine analogue 25 (RS194B). Compound 25 displayed improved in vitro reactivation kinetics with remarkable low toxicity
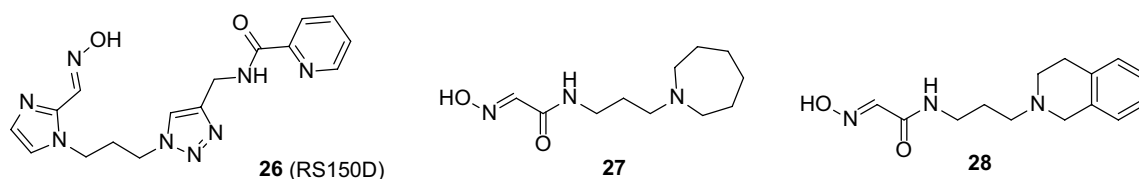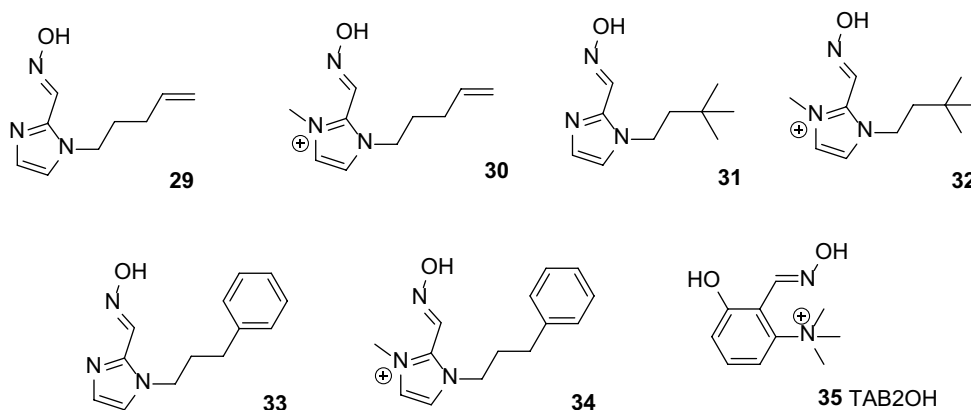
Fig. 12 Uncharged reactivators efficient against tabun-inhibited *h*AChE and *h*BChE (Kovarik et al. 2013)

Fig. 13 Effective imidazole aldoximes used in "catalytic bioscavenger system" (Sit et al. 2014)

in the mouse model after i.m. administration compared to **23** and 2-PAM (Fig. 11) (Radić et al. 2012). Notably, no correlation to asoxime nor obidoxime was performed.

The valuable property of these amidoximes (**23** and **25**) lies in their ability to form protonation equilibrium around two ionizable groups, an oxime group and amine group, affording coexistence of charged, zwitterionic and uncharged forms around physiological pH values. The zwitterionic form has a high chance to reactivate OP-inhibited AChE, while the uncharged form can easily cross the BBB (Sit et al. 2011; Radić et al. 2012). Furthermore, reactivator **25** showed very good antidotal profile within in vivo assessment on mice exposed to the subcutaneous OPs (VX, sarin, cyclosarin, paraoxon and tabun). Authors of the study also concluded that reactivator **25** displayed significant improvement in reactivation potency when compared to oxime **23** and 2-PAM, especially in the combination with prophylactic and post-exposure treatment (Radić et al. 2012).

Reactivation ability of compounds **23** and **25** supported the suggestion that reactivators geometry is important criterion (Kovarik et al. 2013). While uncharged reactivators **23** and **25** exerted high reactivation potency for against sarin-, cyclosarin- and VX-inhibited *h*AChE, their potency against tabun-inhibited *h*AChE was rather poor (Kovarik et al. 2013). Ongoing study involved 29 novel uncharged oximes tested for their ability to reactivate tabun phosphylated *h*AChE and comparing them to three standards, diacetyl-monoxime (DAM), monoisonitrosoacetone (MINA) and 2-PAM (Shih et al. 2010; Skovira et al. 2010). In addition,

reactions with phosphylated *h*BChE and two mutant *h*AChE were also established. Imidazole aldoxime **26** (RS150D) was highlighted to be the most potent reactivator of tabun-inhibited *h*AChE. Some other oximes containing bulkier moieties such as azepane (**25** and **27**) or tetrahydroisoquinoline (**28**, Fig. 12) also displayed some reactivation potential for tabun-inhibited *h*BChE. This finding was explained by better fitting of **25**, **27** and **28** into the active site of *h*BChE which is more bulkier compared to *h*AChE active site to accommodate these ligands (Fang et al. 2011; Kovarik et al. 2013).

Refining the structural features of previously published series, novel imidazole aldoximes analogues were developed as potent reactivators of *h*BChE inhibited by paraoxon, sarin, cyclosarin and VX-agent. All of the compounds were based on imidazolium aldoximes with an alkyl/alkenyl chain (**29–32**) or with aromatic system (phenyl; **33** and **34**; Fig. 13) (Sit et al. 2014). Study also exploited reactivation capability of TAB2OH (**35**) in combination with purified *h*BChE as efficient bioscavenger to potentiate OP-intoxication treatment and reduce the amount of required *h*BChE (Radić et al. 2013). This strategy has already been proposed more than two decades ago as a concept of catalytic bioscavenging. Several enzymes have been considered to act as OPNA-degrading enzymes (e.g. paraoxonase-1, erythrocyte and liver prolidases, human liver senescence marker, cholinesterases mutants) (Kovarik et al. 2010; Nachon et al. 2013).

More recently, Wei and co-workers suggested dual binding site strategy for imidazole aldoximes. This approach

consisted of introducing bulky PAS ligand attached by a methylene tether to the reactivation moiety—imidazole aldoximes. Accordingly, PAS ligand is able to ensure the anchoring of the compound to the active site. Novel eight uncharged derivatives **36–43** (Fig. 14) were developed with the PIQ or TIQ as the PAS ligands connected to the imidazole aldoxime through flexible alkyl spacer of different length. Compounds **36–43** were expected to cross BBB due to their high lipophilicity (calculated log P). The biological evaluation consisted of only in vitro reactivation of the sarin-, tabun- and VX-inhibited hAChE (Wei et al. 2014). The most efficient reactivators among imidazole aldoximes were **42** and **43**, being as efficient as methoxime or asoxime against sarin-inhibited hAChE. Compound **43** exerted the highest reactivation ability for VX-inhibited hAChE, comparable to asoxime or obidoxime. Improved reactivation ability for tabun-inhibited hAChE was achieved with **43** when compared to obidoxime and trimedoxime (Wei et al. 2014).

Based on the requirements for centrally active AChE reactivators and meeting the criteria for drug-like properties (Lipinski et al. 2001; Kuca et al. 2006; Wager et al. 2010), study by M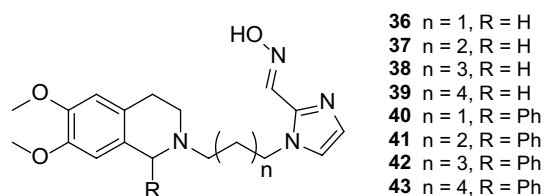cHardy aimed to the synthesis and characterization of the two new series of reactivators containing either amide-oxime moiety (**44** and **45**) with the variety of n-methylene linkers and PAS ligands or bearing the keto-oxime group (**46–48**). All these compounds were assayed in vitro to reactivate cyclosarin-inhibited hAChE. Some compounds **44–48** (Fig. 15) possess substantial reactivation activity compared to MINA and favourable physiochemical characteristics for CNS drugs (McHardy et al. 2014).
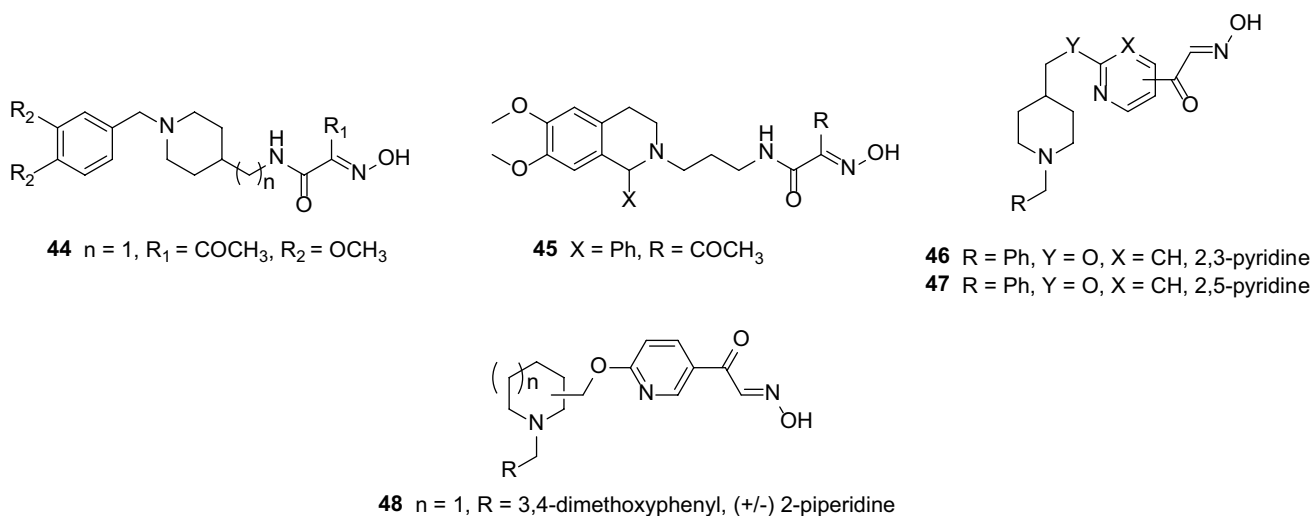
Retro-structural analysis applied to 2-PAM led to novel compounds containing amidine core connected with oxime functional group (Kalisiak et al. 2011, 2012). It was presumed that during the physiological conditions such amidine–oximes exist in protonated form mimicking charged pyridinium moiety of 2-PAM. Therefore, this might facilitates efficient binding via cation-π interactions in the CAS of AChE. In addition, protonated amidine group could potentiate electron-withdrawing effect of the oxime group and thus allow formation of the oximate anion plus zwitterionic form of molecule (Scheme 4) (Kalisiak et al. 2011, 2012).

Firstly, study describing five novel amidine-oxime reactivators (**49–53**) was published. The ability of these compounds to reactivate OP-inhibited hAChE/hBChE was compared to 2-PAM and monoisonitrosoacetone (MINA) (Shih et al. 2010; Skovira et al. 2010) in vitro and also in vivo. The lipophilicity (C Log P) and pK_a parameters were determined as well. pK_a value for **51** was found around 8.0 which is consistent with the ideas for efficient oximes 7.5–8.0 (Gray 1984). Calculated C Log P suggested increasing lipophilicity in relation to the bulkiness of the attached alkyls, assuming better BBB penetration. In vitro reactivation capability was performed on the surrogates of nerve agents (Sp-GBC, Sp-GF-SMe, Sp-GAC) plus
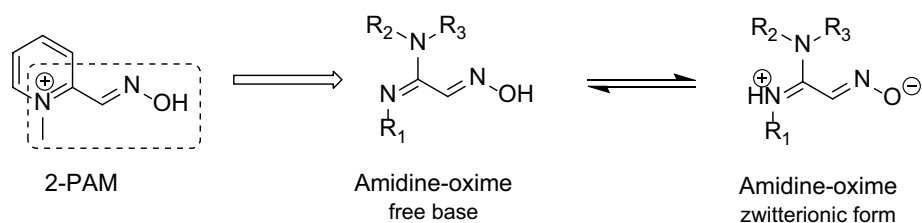
Fig. 14 Imidazole aldoxime reactivators with TIQ or PIQ PAS ligand (Wei et al. 2014)

36	n = 1, R = H
37	n = 2, R = H
38	n = 3, R = H
39	n = 4, R = H
40	n = 1, R = Ph
41	n = 2, R = Ph
42	n = 3, R = Ph
43	n = 4, R = Ph

44 n = 1, R$_1$ = COCH$_3$, R$_2$ = OCH$_3$

45 X = Ph, R = COCH$_3$

46 R = Ph, Y = O, X = CH, 2,3-pyridine
47 R = Ph, Y = O, X = CH, 2,5-pyridine

48 n = 1, R = 3,4-dimethoxyphenyl, (+/-) 2-piperidine

Fig. 15 Most potent compounds for cyclosarin-inhibited hAChE from the study of McHardy (McHardy et al. 2014)
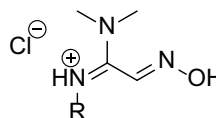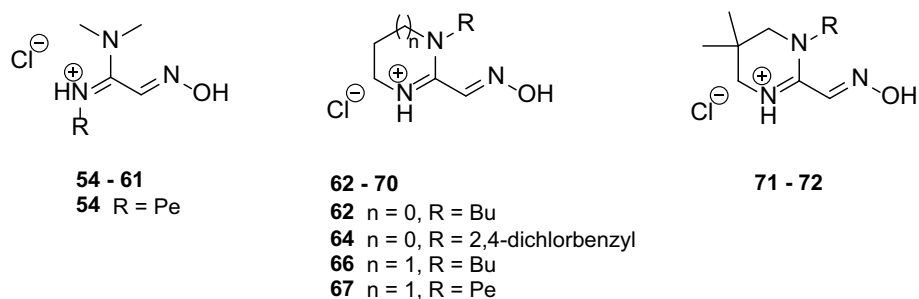
pesticide echothiophate and in vivo with the model of nerve agent (*Sp*-GB-Am) (Barakat et al. 2009; Gilley et al. 2009). All compounds showed superior efficiency compared to MINA. Their reactivation potency increased with the alkyl chain length (from H to *N*-butyl). The most efficient compounds were **51**–**53** yet they did not exceeded reactivation potency of 2-PAM in vitro. The study suggested that they are able to reach more effective concentrations in the brain than 2-PAM due to the higher lipophilicity (Kalisiak et al. 2011).

Subsequent study combined structural features of amidine–oximes into acyclic (including **52**–**53** and eight novel derivatives **54**–**61**) and cyclic compounds (**62**–**70** and **71**–**72**) (Fig. 16) (Kalisiak et al. 2012). These derivatives were biologically evaluated exerting greater reactivation efficiency than MINA. None of the tested compounds surpassed reactivation power of 2-PAM in vitro. On the other hand, mice pre-treated with two amidine–oximes (**53** and **66**) displayed some positive effect against lethal doses of *Sp*-GB-Am (0.08 mg/kg in 0.25 mL/mouse in saline, i.p.). Moreover, animal groups pre-treated with **52**–**53**, **54**, **62**, **64**, **66** and **67** showed only minor signs of CNS toxicity (Kalisiak et al. 2012).

Based on the results of molecular modelling studies, non-oxime hydrazone reactivators (Petronilho et al. 2015) were proposed as 2-PAM structural mimics (Delfino and Figueroa-Villar 2009). The study disclosed synthesis

of nine novel compounds (**73**–**81**, Fig. 17) which were determined for their reactivation potency against paraoxon-inhibited *Ee*AChE in vitro (Petronilho et al. 2015). Unfortunately, results indicated that hydrazones as well as guanylhydrazones are not effective against paraoxon intoxication thus suggesting their inappropriate nucleophilic character. On the other hand, **73**–**81** revealed interesting AChE inhibition profile potentially applicable for the treatment of Alzheimer's disease or Myasthenia gravis (Komloova et al. 2010; Zemek et al. 2014). The hydrazones and guanylhydrazones were found less acidic than oximes and thus have only minor capacity to be deprotonated for successful reactivation at pH 7.4 in the AChE active site (Petronilho et al. 2015).

Hydroxylamine nucleophile as an oxime alternative to counteract OPs exploiting ab initio quantum chemical calculations and steered molecular dynamic studies were also presented (Lo and Ganguly 2014). The calculations showed that the newly designed compound *N*-(pyridine-2-yl)hydroxylamine (**82**, Fig. 18) could be more effective in reactivating tabun-inhibited AChE than charged reactivators or uncharged reactivator 3-hydroxy-2-pyridinealdoxime, with activation barrier around 1.7 kcal mol^{-1}, which is by 7.2 kcal mol^{-1} lower than that found for trimedoxime (Kassa et al. 2005). However, the docking analysis showed that the charged bis-quaternary pyridinium oximes have greater binding affinity to the active-site gorge of *h*AChE

76 R = H
77 R = NO$_2$
78 R = Cl
79 R = CH$_3$
80 R = N(CH$_3$)$_2$
81 R = OH
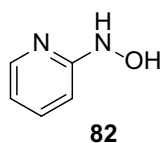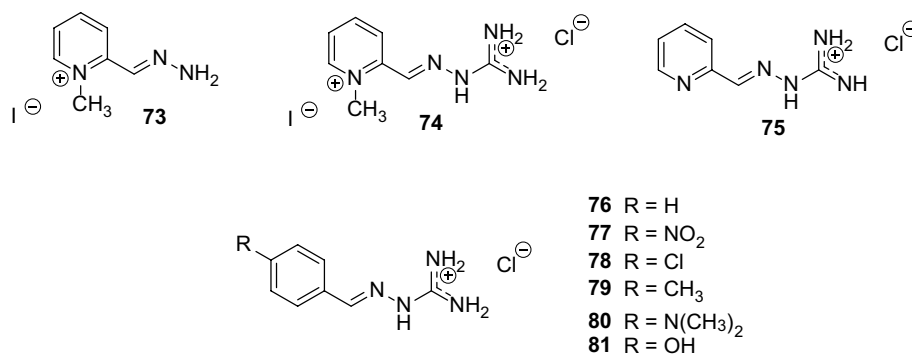
Fig. 18 *N*-(pyridine-2-yl)hydroxylamine (Lo and Ganguly 2014)

when compared to compound **82**. These contradictory findings can be counterbalanced by suitable log*P* values for **82**-derived compounds supporting their ability to penetrate through the BBB. Biological studies showed that the toxicity of hydroxylamine **82** is similar to the methoxime, trimedoxime and obidoxime. Kinetic analysis and structural calculations for hydroxylamine **82** suggested interesting hit for further development of OPs antidotes (Lo and Ganguly 2014).

Other effort in the development of new chemical entities replacing oxime compounds was presented by Bhattacharjee et al. (Bhattacharjee et al. 2015). These pharmacophores were designed by in silico methods using model of tabun-inhibited eel AChE (Bhattacharjee et al. 2010). As a result, five novel non-oximes reactivators were selected from the databases (Maybridge and ChemNavigator) which showed efficacy for DFP-inhibited eel AChE comparable to 2-PAM (Bhattacharjee et al. 2012). Through the screening of the in-house WRAIR-CIS database, 67 compounds were further selected for in vitro studies on DFP-inhibited

eel AChE. Four of them (**83–86**, Fig. 19) with the highest efficacy were suggested for in vivo testing on guinea-pigs poisoned by DFP. Leading compound **83** demonstrated the best overall therapeutic efficacy similar to 2-PAM against DFP-inhibited AChE. Certainly, this compound needs further investigation to assess its full therapeutic potential with biological characteristics (e.g. pharmacokinetics, pharmacodynamics) (Bhattacharjee et al. 2015).

Recently, Mannich phenols and general bases were introduced as new class of non-oxime reactivators (Fig. 20) (Katz et al. 2015). From the library screening, the identified bioactive compounds were related to the drug amodiaquine (ADQ, **87**), well-known antimalarial agent (Burckhalter and Tendick 1948). ADQ was more active than 2-PAM in reactivation of DFP and paraoxon-inhibited AChE; however, hepatotoxicity and agranulocytosis issues prevented this compound from further development. Screening of novel ADQ analogues revealed that the most important fragment responsible for reactivation is 4-amino-2-(diethylaminomethyl)phenol (ADOC, **88**), basically ADQ (**87**) without hydrophobic anchor. ADOC (**88**) displayed in vitro reactivation potency towards paraoxon-, DFP-, sarin surrogate (NIMP)- and soman analogue (SIMP)-inhibited *h*AChE (Li et al. 2001; Gilley et al. 2009; Meek et al. 2012). Since the phenol is a leaving group within AChE phosphylation (in the case of paraoxon), the phosphonylation could be reverted with excess of suitable phenols such as Mannich phenols (Epstein et al. 1964),
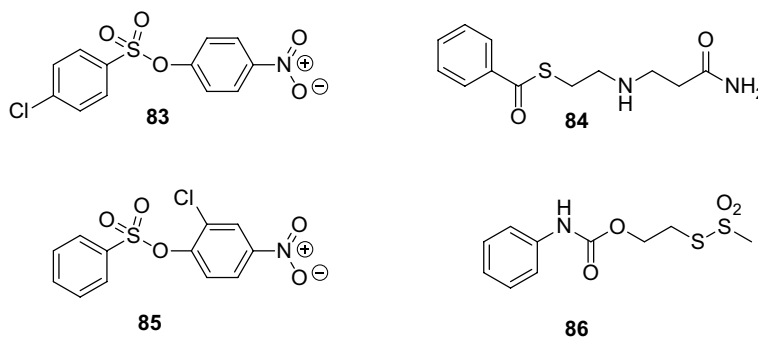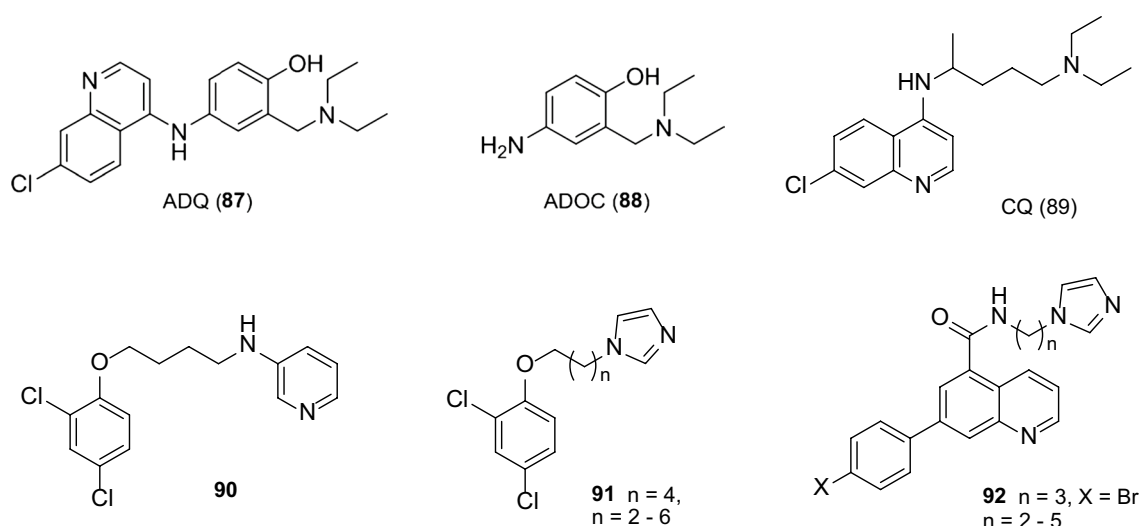
Fig. 20 Amodiaquine (**87**), Mannich phenol **88**, chloroquine (**89**) and novel compounds **90**–**92** (Katz et al. 2015)

Fig. 21 Trimedoxime and K048 (**93**) (Kuca et al. 2003)
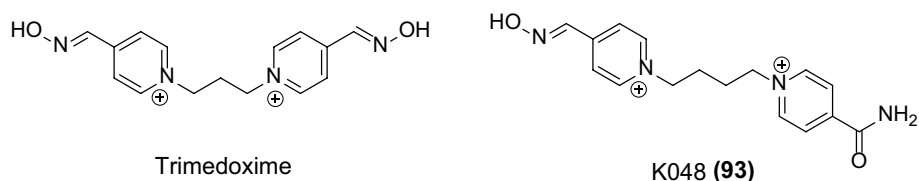
nitrophenols (Muthukrishnan et al. 2012) and coumarins (Briseño-Roa et al. 2006; Orhan and Gulcan 2015). Moreover, chloroquine (CQ, **89**), another antimalarial drug and less toxic ADQ analogue without phenol moiety, was also found to exert reactivating ability (Michaelides et al. 2011). 9-Chloroquinoline ring showed pivotal role in conferring reactivation ability and its derivatization resulted in hybrid compounds **90**–**92** (Katz et al. 2015). The most active compounds and their reactivation profiles were chosen after further screening and SAR studies. Leading analogues **88**, **91** and **92** were assayed in vivo on mouse model subcutaneously exposed to the lethal dose of DFP (3 mg kg^{-1}; 95 % of mortality). Highly soluble hybrid **88** was administered i.p. assaying both pre- and post-exposure treatments. Significant neuromuscular relaxing side effects of analogue **91** were observed. On the contrary, conjugate **92** was administrated only p.o. as a prophylactic agent. Number of survivors and cholinesterase activity in all tissues were measured after 24 h and compared to 2-PAM. Cholinesterase activity was restored in all tissues including CNS in case of administration of compounds **88** and **92**. Administration of 2-PAM resulted in the lower AChE activity compared to the levels of AChE after pre- and post-exposure administration of reactivator **88**. Additionally, relief of neurological symptoms were observed in case of **88** and **92** contrary to the 2-PAM (Katz et al. 2015).

Peripheral active site ligands

Within the effort to develop more potent reactivator, some studies were focused on the investigation of new peripheral ligands attached through the linker to the reactivation moiety (in the most cases oxime group). The main purpose for introducing PAS ligand is improving the binding affinity to the PAS of the enzyme. Other rationale is directed to the diverse conformation of AChE/BChE enzymes in order to find highly selective reactivator.

Trimedoxime analogue K048 (**93**) with carbamoyl group (Fig. 21) was found to be more efficient at reactivating tabun-inhibited *h*AChE in vitro than trimedoxime itself. The plausible explanation for this phenomenon stems from the fact that K048 has better affinity towards OP-inhibited *h*AChE which is mediated by carbamoyl moiety through the hydrogen bonds to the PAS (Kuca et al. 2003).

The inspiration for the potent reactivators with peripheral site ligand (PSL) can be found from the research of AChE inhibitors. Indeed, PIQ was the first presented in the study dealing with in situ click chemistry of AChE inhibitors (Krasiński et al. 2005). PIQ showed high binding affinity with orientation towards PAS. Additionally, newly in situ-clicked multivalent inhibitors displayed a surprisingly low preference for PIQ stereoisomers (Krasiński et al. 2005). PIQ was exploited as a valuable tool in many
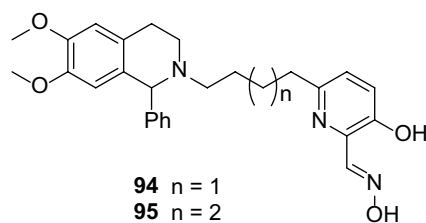
Fig. 22 In vitro efficient uncharged reactivators (Mercey et al. 2011)

ongoing studies studying novel AChE inhibitors or reactivators. Initially, PIQ was used as an attachment for the oxime **14** revealing first uncharged AChE reactivator **94–95** (Fig. 22) (Mercey et al. 2011; Saint-André et al. 2011). This study further showed that there exists balanced binding affinity which is high for the phosphylated and low for the uninhibited enzyme. Based on the results from the molecular docking simulation, authors suggested that the best candidates should have linker attached in the position 6 of 3-hydroxy-pyridine-2-aldoxime **14**. The fact that PAS is located approximately 15 Å above the active site predicted ideal length between PSL and oxime moiety corresponding to alkyl chain composed of 4–5 methylene units. Further investigation in the optimal linker and its positioning was described elsewhere (Mercey et al. 2012a). Oximes **94** and **95** were tested in vitro against tabun- and VX-inhibited *h*AChE. Their reactivation parameters were compared to the known oximes (obidoxime, asoxime, trimedoxime and HLö-7). Oximes **94** and **95** showed superior or similar in vitro ability to reactivate the enzyme compared to these standards. It was disclosed that there exists trade-off between longer connecting linker which allows better binding within enzyme but less reactivation rate (Mercey et al. 2011).

Based on the promising results obtained in the pioneering work leading to **94** and **95**, seven uncharged AChE reactivators were further reported (Renou et al. 2013). Retaining the fragment **14** connected through the alkyl tethers of various length to the different peripheral site ligands with the high affinity towards the OP-inhibited *h*AChE (Muñoz-Ruiz et al. 2005; Alonso et al. 2005; Khorana et al. 2012). These reactivators **96–102** (Fig. 23) were assayed in vitro against tabun- and VX-inhibited *h*AChE, and their reactivation abilities were compared to 2-PAM, obidoxime and asoxime. Results showed that only **101** possesses equivalent reactivation potency to 2-PAM. All other derivatives were found less efficient in comparison with the bis-pyridinium aldoximes (obidoxime, asoxime) (Renou et al. 2013).

In the follow-up study, additional uncharged reactivators were presented for OP-inhibited *h*AChE and OP-inhibited *h*BChE which were used as efficient pseudo-catalytic

bioscavengers (Kovarik et al. 2010; Radić et al. 2013; Renou et al. 2014). Again, 3-hydroxy-pyridine-2-aldoxime was used in combination with tryptoline which is moderate affinity binder of both *h*AChE and *h*BChE (Costagli and Galli 1998). The tryptoline-based compounds **103–105** (Fig. 24) were tested in vitro for the reactivation of the VX-inhibited *h*BChE. The results showed that they were potent reactivators for VX-inhibited *h*BChE, specifically oxime **104** was the best reactivator known to date with efficiency comparable to obidoxime. Explanation for the good affinity is probably due to a formation of π–π interaction between tryptoline moiety and Tyr332 at the entrance to *h*BChE active site (Wandhammer et al. 2013). In addition, all these oximes showed reactivation potency of OP-inhibited *h*AChE as well. Particularly, compound **105** was as efficient as asoxime for VX-inhibited *h*AChE in vitro. Compound **104** exerted similar activity as trimedoxime for tabun-inhibited *h*AChE (Renou et al. 2014).

Scaffolds composing of 3-hydroxy-pyridine-2-aldoximes or 3-hydroxy-pyridine-2-amidoximes conjugated to the well-known PAS ligand 1,2,3,4-tetrahydroacridine (tacrine) were reported (Colletier et al. 2006; Rydberg et al. 2006; Kliachyna et al. 2014). Authors studied the influence of various linkers on the different positions of pyridine ring. Four novel amidoximes (**106–109**) and five aldoximes (**110–113**) were reported (Fig. 25) (Kliachyna et al. 2014). Reactivation potencies of tacrines **106–113** were evaluated in vitro against tabun-, ethyl-paraoxon- and VX-inhibited *h*AChE in comparison with reactivation kinetics to 2-PAM, obidoxime, trimedoxime, asoxime and HLö-7. Compounds **107** and **109** were found inefficient in replenishing AChE activity. On the other hand, the rest of series displayed in vitro similar or superior reactivation ability to the standard reactivators highlighting compound **112**. This oxime showed higher efficiency in reactivation of VX-inhibited *h*AChE compared to 2-PAM, obidoxime, trimedoxime and asoxime but less than HLö-7. Moreover, **112** exhibited the highest potency for tabun-inhibited *h*AChE than used bisquaternary reactivators in vitro. Oxime **112** might be also useful in intoxication with ethyl-paraoxon as it showed comparable activity to obidoxime and HLö-7 (Kliachyna et al. 2014).

In vivo assessment of these uncharged cholinesterase reactivators is currently awaited by the scientific community to prove their biological effectiveness. Two of these uncharged reactivators **94** (GM113) and **115** (GM508; Fig. 26) have already been biologically evaluated towards VX-inhibited AChE in mice. Mice were intraperitoneally treated with asoxime, 2-PAM, **94** and **115** directly 1 min after s.c. intoxication of VX (LD50 = 22 μg/kg). Asoxime showed highest reactivation potential 4-fold higher than the rest of three reactivators at the same concentration (100 μmol/kg bw.). Moreover, poor solubility of
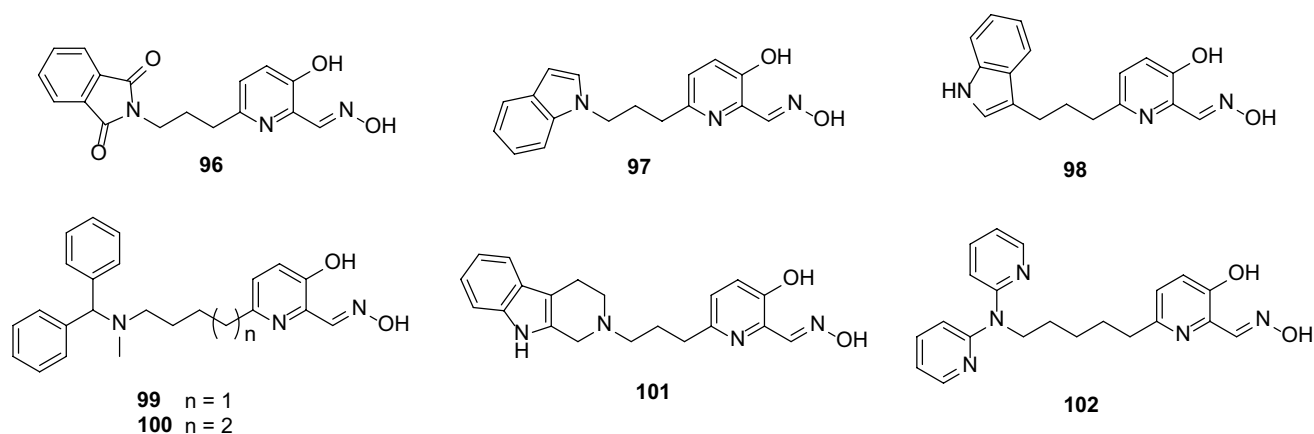
Fig. 23 3-Hydroxy-pyridine-2-aldoxime based reactivators of AChE (Renou et al. 2013)
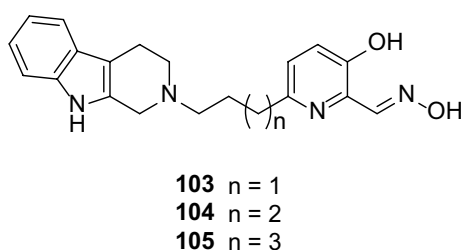

Fig. 24 3-Hydroxy-pyridine-2-aldoxime in combination with tryptoline representing PAS ligand (Renou et al. 2014)

reactivators **94** and **115** resulted in lower bioavailability and thus lower plasma levels (Kipp 2004; Mercey et al. 2011; Calas et al. 2016).

Compounds **116–118** (Fig. 27) were proposed as novel class of reactivators combining charged 4-PAM moiety attached through the ethylene glycol linkers of different length to the non-ionic benzhydryl-piperidine moiety (Ohta et al. 2006; Kwon et al. 2007; Shih et al. 2009; de Koning et al. 2011a). Authors suggested that this PAS ligand should increase lipophilicity, thus improving permeability through BBB and enhance reactivating capacity of constructs compared to parent pyridinium-4-aldoximes **119–121** (Fig. 27) (de Koning et al. 2011a). The reactivation potencies of these compounds were evaluated against sarin-, tabun- and VX-inhibited hAChE in vitro compared to the intermediates **119–121**, 4-PAM and asoxime. For the sarin- and VX-inhibited hAChE, **116–118** showed superior reactivation potency than **119–121** or 4-PAM but
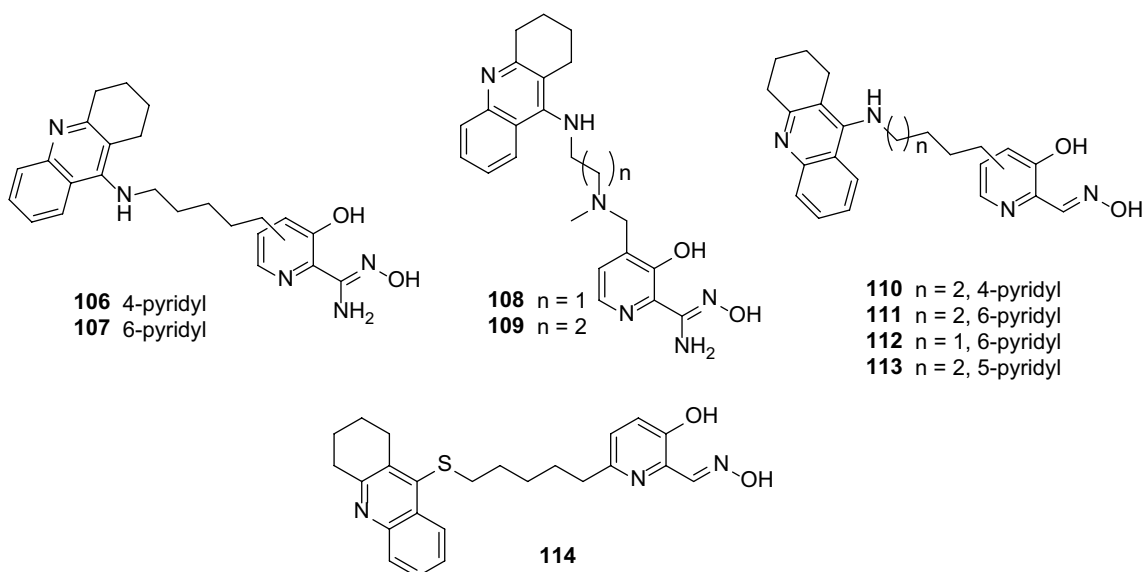

Fig. 25 3-Hydroxy-pyridin-2-amidoximes (**106–109**) and 3-hydroxy-pyridin-2-aldoximes (**110–114**) attached to tacrine moiety (Kliachyna et al. 2014)
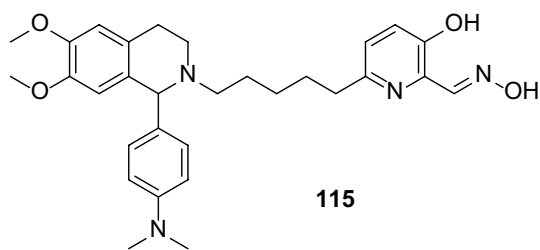
Fig. 26 Uncharged reactivator 115 (GM508) based on 3-hydroxy-pyridin-2-aldoxime

Fig. 29 General structure of phenoxyalkyl pyridinium-4-aldoximes (Chambers et al. 2013)

slightly lower efficacy than asoxime pointing out to 118 being the most promising candidate in the series. For the tabun-inhibited *h*AChE, it was found out that only oxime 118 exhibited efficiency equal to the 4-PAM (de Koning et al. 2011a).

Taking into account, the results from previous study, ketoximes with benzhydryl-piperidine 122–124 (Fig. 28) were proposed (de Koning et al. 2011b). These compounds were evaluated against sarin-, tabun- and VX-inhibited *h*AChE compared to the parent oxime 125 and fragments 126–128 (Fig. 28) in vitro. Results showed significantly higher reactivation potency in case of 122–124 compared to 125–128. Interestingly, no significant influence in the linker length was observed. Conjugate 123 yielded with the best reactivation potency against sarin-inhibited *h*AChE. The reactivation ability for all the hybrids 122–128 against VX-AChE complex were in similar range. In case of tabun

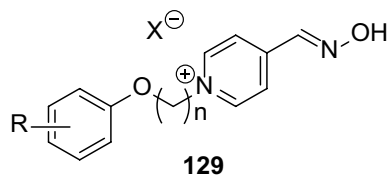inhibition, no reactivation potency was observed. The reactivation activity increase can be observed between 116–118 and 122–124. Authors suggested positive influence of dual binding site strategy applied to ChE reactivators and improvement of reactivation potency of molecules with poor efficiency (de Koning et al. 2011b).

A set of 35 phenoxyalkyl pyridinium aldoximes of general structure 129 (Fig. 29) was designed with improved lipophilicity to presumably cross BBB in higher extent (Chambers et al. 2013). Phenoxyalkyl pyridinium-4-aldoximes 129 combine 4-PAM attached through methylene tether (*n* = 3–6) to the differently substituted phenoxy moieties. These aldoximes were tested in vitro for their reactivation ability on surrogates of sarin- and VX-inhibited rat brain AChE in comparison with 2-PAM and trimedoxime (Ohta et al. 2006; Meek et al. 2012). Compounds proving efficacy higher than 40 % (11 out of 35) proceeded to in vivo evaluation using rats. Regardless of the compounds pharmacokinetics, oximes were administrated i.m. 1 h after

Fig. 27 Benzhydryl-piperidine attached to charged pyridinium-4-aldoximes 116–118 and parent pyridinium-4-aldoximes 119–121 (de Koning et al. 2011a)

116 n = 1
117 n = 2
118 n = 3

119 n = 1
120 n = 2
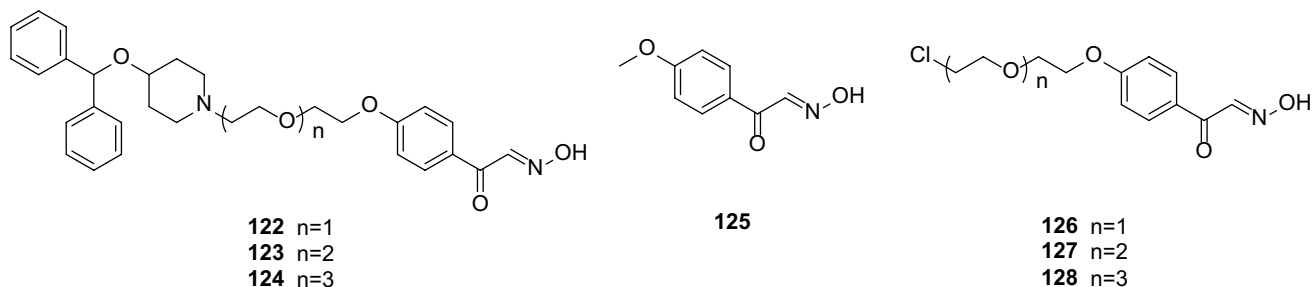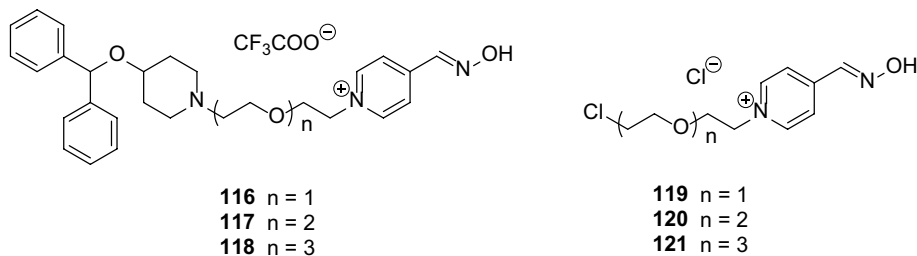121 n = 3

122 n=1
123 n=2
124 n=3

125

126 n=1
127 n=2
128 n=3

Fig. 28 Benzhydryl-piperidine attached to non-charged pyridin-4-aldoximes 122–124, parent pyridin-4-α-ketoaldoximes 125 and intermediates with ethylene glycol linkers 126–128 (de Koning et al. 2011b)
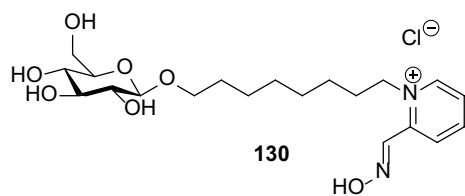
Fig. 30 The most potent sugar-oxime reactivator **130** (Garcia et al. 2010)

131 - 134
135 X = Cl, R = -CH₂COOEt
136 X = Cl, R = -COOEt

137 - 142

Fig. 31 *N*-Thiazolylacetamide 4-PAMs **131**–**136** and 3-PAMs **137**–**142** derivatives (Valiveti et al. 2015a)

143 R = -Ph-4-OMe
145 R = -Ph-4-CN
146 R = -Ph-4-COOMe
147 R = -CH₂-Ph
148 R = -(CH₂)₅-COOH

144 R = -Ph

Fig. 32 Mono-quaternary pyridinium aldoximes with arenylacetamides in the side chain **143**–**148** (Valiveti et al. 2015b)

sarin surrogate i.p. administration (80 % brain AChE inhibition). Three of these novel oximes showed 15–25 % brain AChE reactivation within 30 min after their administration (Chambers et al. 2013).

Interesting strategy was proposed via combining saccharide structure with reactivation moiety (Garcia et al. 2010). The rationale of the study was supported by the GLUT1 transporter that can be found on the brain membrane. It is relatively non-specific and able to transport variety of hexoses and glucose-conjugates. Evidence of BBB penetration of the sugar-oxime conjugates have already been reported (Rachaman et al. 1979; Heldman et al. 1986). Based on these presumptions, 14 saccharide-oximes including six novel compounds and eight previously reported compounds were studied (Rachaman et al. 1979; Heldman et al. 1986; Garcia et al. 2010). Abilities of these saccharide-oximes were evaluated on DFP-, paraoxon-, sarin- and VX-inhibited *h*AChE and *h*BChE in vitro. After 20 min, oxime **130** (Fig. 30) was nearly as effective as 2-PAM for sarin- and VX-inhibited *h*AChE with 80 and 20 % recovery of enzyme activity, respectively. 2-PAM saccharide analogues were more effective than 3-PAM or 4-PAM ones. Eight-methylene tether linker was suggested to be optimal for conjugation of 2-PAM and glucose allowing the glucose to interact with PAS of *h*AChE (Garcia et al. 2010).

Mono-quarternary pyridinium aldoximes, namely 12 analogues of 4-PAM (**131**–**136**) or 3-PAM (**137**–**142**), attached to substituted *N*-thiazolylacetamide moiety were developed (Fig. 31) (Valiveti et al. 2015a). Thiazole is largely utilized chemical scaffold engaged in variety of drug candidates with a capability of binding to specific enzymes acting as reversible inhibitors, e.g. in some neurological disorders (Gil et al. 2009; Bulic et al. 2010; Lee et al. 2012; Valiveti et al. 2015a). *N*-Thiazolylacetamide moiety was introduced to provide non-covalent interaction with the aromatic residues within the PAS of *h*AChE. Additionally, acetamide linker may facilitate proper orientation of the reactivating moiety due the electrostatic interactions inside the narrow AChE gorge. All compounds were in vitro evaluated for their reactivation potency against sarin-, *O*-ethylsarin- and VX-inhibited *h*AChE including pharmacokinetic parameters and compared to 2-PAM and
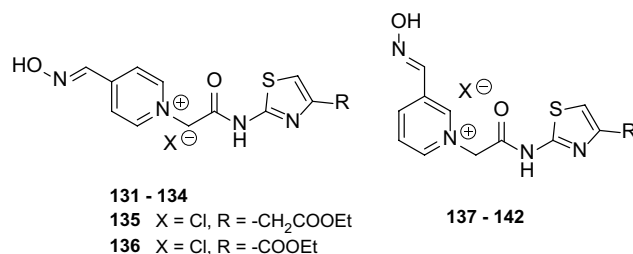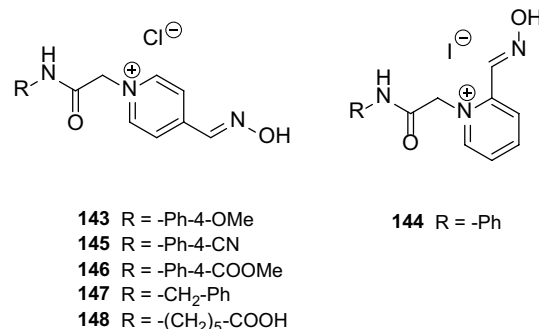
obidoxime. 4-PAM-derived *N*-thiazolylacetamides were found more active than 3-PAM analogues. This phenomenon can be explained by calculated pK_a values ranging between 8 and 8.5 (for 4-PAM) and 8.5–8.9 (for 3-PAM). The leading compounds of the study **135** and **136** did not overwhelm capability of obidoxime in the case of all tested nerve agents. The highest reactivation potency showed *N*-thiazolylacetamides bearing ester moiety suggesting its proper orientation towards phosphylated AChE. Docking studies confirmed importance of hydrogen bond interactions between various amino acid residues and the amide linker or ethoxycarbonyl substituent (Valiveti et al. 2015a).

Encouraged by the results from previous study, other series of novel mono-quaternary pyridinium aldoximes with attached arenylacetamides was presented (Valiveti et al. 2015b). Prepared oximes were evaluated in vitro for sarin-, tabun- and VX-inhibited *h*AChE compared to the 2-PAM and obidoxime. Results showed that all oximes revealed significant potency in reactivating sarin- and VX-inhibited *h*AChE; however, reactivation potency against tabun-inhibited *h*AChE was only marginal. Six compounds in the series **143**–**148** (Fig. 32) were highlighted with excellent ability to reactivate sarin-inhibited *h*AChE, while analogues **145**–**147** were the best against VX-inhibited *h*AChE exceeding reactivation potencies of 2-PAM and obidoxime (Valiveti et al. 2015b).
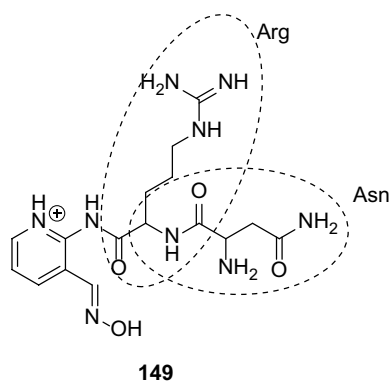
Fig. 33 2-Amino-pyridin-3-aldoxime-(Arg-Asn) **149** (Chadha et al. 2015)

In silico research of the 2-amino-pyridine-3-aldoxime-dipeptides as potential AChE reactivators was based on the previous observations suggesting its improved AChE-reactivation profile (Tiwari et al. 2012; Chadha et al. 2015). Study involved in silico methods such as molecular docking, molecular dynamics and density functional theory to get insight into the binding mode in the AChE active site with particular emphasis to the specificity and selectivity. Results showed that oxime **149** (Fig. 33) interact with residues of the active site and PAS region, namely Tyr72, Tyr124, Trp286, Tyr341 and His447 and additional Glu285, Ser293 and Phe295 contributing to accommodation of the oxime moiety within the active site. Therefore, synthesis of these molecules and their in vitro/in vivo studies might be the matter of further interest (Chadha et al. 2015).

In addition, PAS binders included *N*-benzylpiperidine (**150**), tacrine (**151**), phenyl-tetrahydroisoquinoline, tetrahydroisoquinoline (**152–153**) as well as heteroaryl ketooximes with substituted *N*-piperidine or *N*-pyrrolidine

(**154–156**) were proposed (Fig. 34). Most potent compounds of this series were described in the section with CAS ligand (Fig. 15) (McHardy et al. 2014).

Computational study which designed new reactivators capable to reverse ageing process of OP-inhibited AChE as well as reactivate aged AChE was also published (Chandar et al. 2014). Steered molecular dynamics simulation and molecular docking methods suggested that newly designed dimethyl(pyridin-2-yl)sulfonium (**157**) is more efficient in the alkylation process of the phosphorus atom of the "aged" AChE-OP adduct than earlier reported *N*-methyl-2-methoxypyridinium **158** (Topczewski and Quinn 2013). Combination of these scaffolds with pyridin-2-aldoxime resulting in compound **159** that could also serve as reactivator for inhibited AChE. Calculated ClogP of 3.35 also presumed BBB penetration for analogue **159** (Fig. 35) (Chandar et al. 2014).

Linkers

Whereas several of new CAS and PAS ligands were reported in the past few years, studies concerning modifications of linkers between those two fragments were reported only rarely. More likely, linkers between both anionic sites used in the recent publications were based on the previous knowledge (Musilek et al. 2007b). Ongoing studies in AChE reactivators investigate new SAR between linkers with the aim to find new and more efficient candidate potentially useful for OP poisoning. SAR study focused on modifications of linkers between two pyridinium rings have already been published reviewing the period between 1990 and 2004 (Musilek et al. 2007b). Based on these observations, structural modifications in the bis-quaternary reactivators connected via double-bond (Kuca et al. 2005; Musilek et al. 2007a; Acharya et al. 2009a), xylene
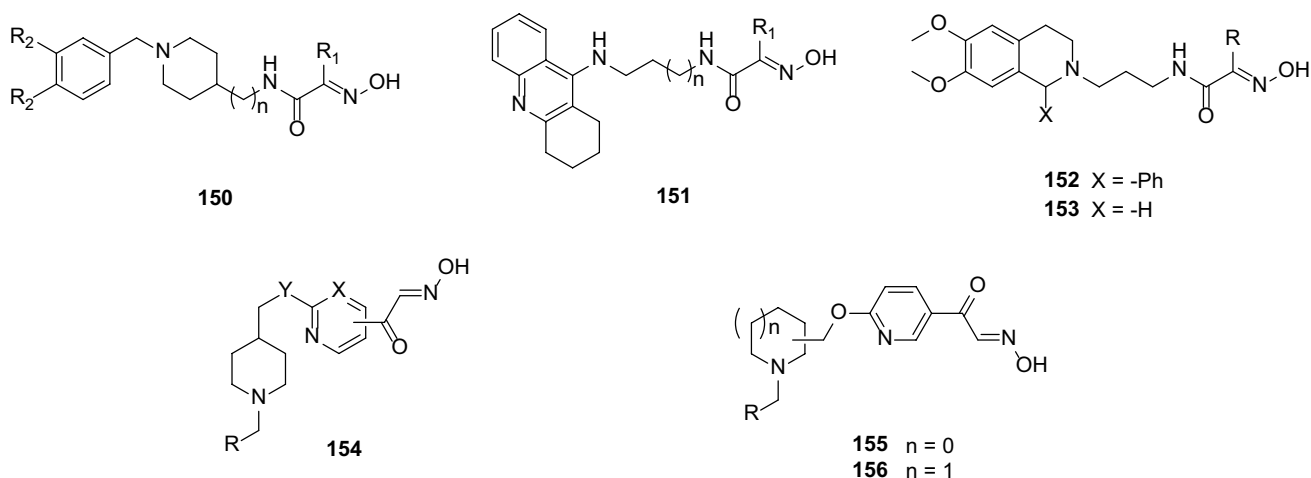

Fig. 34 Common structures of reactivators with various PSL for cyclosarin-inhibited *h*AChE (McHardy et al. 2014)

Fig. 35 Dimethyl(pyridin-2-yl) sulfonium (**157**) conjugated with pyridine-2-aldoxime (**159**) (Chandar et al. 2014); *N*-methyl-2-methoxypyridinium (**158**) (Topczewski and Quinn 2013)
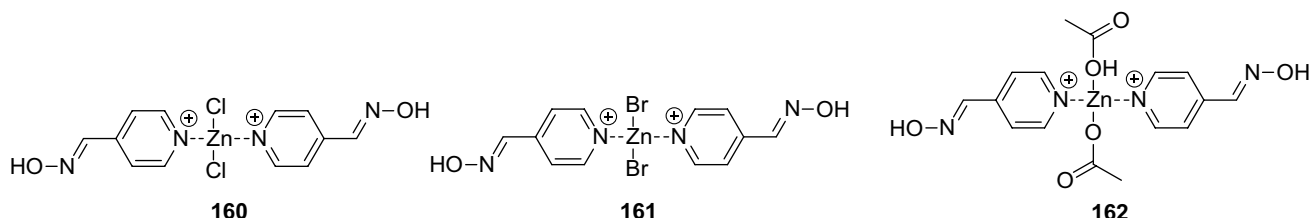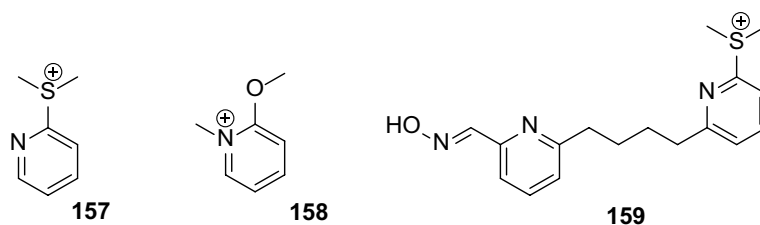

Fig. 36 Bis-pyridinium aldoximes-Zn^{II} complexes (Konidaris et al. 2014)

(Musilek et al. 2005; Acharya et al. 2009a, b; Musilek et al. 2010; Acharya et al. 2011; Gupta et al. 2014; Sharma et al. 2014), and combinations with oxygen analogues have been reported (Kim et al. 2005; Chennamaneni et al. 2005; Srinivas Rao et al. 2006; Oh et al. 2006; Acharya et al. 2008, 2009a, 2010). These modifications have already been discussed in the recent review papers (Mercey et al. 2012b; Sharma et al. 2015).

Interesting strategy has been shown in combination of pyridinium aldoximes using their complexes with Zn^{II} (**160**–**162**, Fig. 36) (Konidaris et al. 2014). Authors took advantage of Zn^{II} being Lewis acid and thus enhancing the nucleophilicity of the oxime moiety in combination with oximate may lead to decrease the pK_a closer to the physiological values. This assumption has already been envisaged before (Bolton and Beckett 1964; Breslow and Chipman 1965; Breslow and Overman 1970). New complexes were tested for reactivation potency against paraoxon-inhibited *Ee*AChE compared to obidoxime and dinuclear complex of the same ligand [Zn₂(O₂CPh)₄(pyridin-4-aldoxime)₂].2-MeCN (Konidaris et al. 2009). The rationale for the design stemmed from molecular modelling survey (Konidaris et al. 2014). Results showed that complexes **160**–**162** are moderately active reactivators highlighting the derivative **160**, however, not reaching the activity of obidoxime. Interestingly, [Zn₂(O₂CPh)₄(4-pyridinealdoxime)₂].2-MeCN (dinuclear complex) displayed no activity. Molecular modelling studies revealed that the mononuclear Zn^{II} complexes possess structural and physical–chemical properties to move across the narrow gorge and arrange accommodation into the active site of the enzyme providing several important interactions with both anionic sites. Poor reactivation potency of Zn-dimers might be explained by more

distant orientation of oximate to P–O bond and formation of stronger interaction to the mid-region of the enzyme (Konidaris et al. 2014).

The latest trends in development of novel AChE reactivators tend to mono-pyridinium or uncharged reactivators bringing also some novelty to the linkers connecting CAS reactivation moieties with PAS ligands as well as exploiting the well-known features from the bis-pyridinium reactivators.

Suggestion that optimal length for the linker lies between 4 and 5 methylenes was proposed (Mercey et al. 2011, 2012a). Nine novel analogues were described (**164**–**172**, Fig. 37) (Mercey et al. 2012a), and their activities were compared to previously reported oximes **94** and **95** (Fig. 22) (Mercey et al. 2011). Structural modifications included variable linker length, attachment of oxime moiety on the pyridine ring and heteroatom insertion into the linker. Their potential was evaluated performing in vitro reactivation for tabun-, ethyl-paraoxon- and VX-inhibited *h*AChE. Overall, the highest reactivation ability goes for analogues 4- or 5-methylenes tether attached to position 6 of the pyridine ring. With this regard, **94** and **95** provided the highest potential for reactivation VX-inhibited *h*AChE, yet all compounds showed superior reactivation ability towards 2-PAM. In case of tabun-inhibited *h*AChE, three novel reactivators **165**, **166** and **169** were more efficient than trimedoxime. Moreover, compounds **164** and **170** displayed similar efficacy to trimedoxime. Three compounds, namely **94**, **95** and **165**, showed reactivation potency to paraoxon-inhibited *h*AChE comparable to obidoxime and trimedoxime (Mercey et al. 2012a).

Researchers from University of Rouen studied reactivation ability of novel tacrine-based reactivators. In this case,
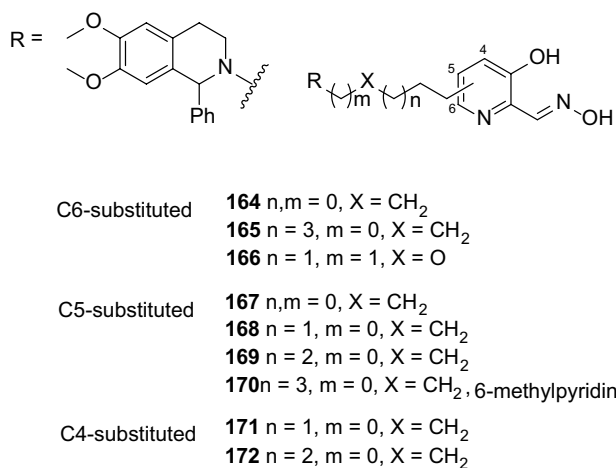
Fig. 37 Conjugates of 3-hydroxypyridinaldoximes and phenyl-tetrahydroisoquinoline connected through the different linkers 164–172 (Mercey et al. 2012a)

tacrine was used as anchor occupying PAS of the enzyme in combination with simple alkyl linkers ranging from 3 to 5 methylenes connected to reactivation moiety. In that case 4 methylene linkers were highlighted (112, Fig. 25) (Kliachyna et al. 2014). Another study reported tryptoline as PAS ligand, highlighting three methylene-tethered analogue (Fig. 24) (Renou et al. 2014). More details regarding these works can be found in the section dealing with CAS or PAS ligands.

Similarly, incorporation of ethylene glycol linkers of different length was used to improve the solubility issues (Figs. 27, 28) (de Koning et al. 2011a, b). In the first series, increase in the length of the linkers enhanced reactivating potential of such charged reactivators (Fig. 27) (de Koning et al. 2011a). In the second subset of non-charged hybrids, no significant influence between length of these linkers and activity was observed (Fig. 28) (de Koning et al. 2011b).

Thiazolylacetamides or phenylacetamides were used as side chain connected to 4-PAM suggesting that acetamide moiety may facilitate proper orientation of the oxime moiety due the electrostatic interactions inside the AChE narrow gorge. Indeed, docking studies demonstrated formation of hydrogen bonding between the amino acids residues of the enzyme and the amide linker which resulted in the favourable orientations of reactivators in the active site of hAChE (Fig. 32) (Valiveti et al. 2015a, b).

Discussion

Herein, we have presented various approaches from different research groups in the search and development of novel and more potent AChE reactivators. One strategy

shifted from well-established and largely exploited compounds bearing aldoxime group to novel nucleophiles bringing in general interesting results. On the other hand, hydrazones and guanylhydrazones analogues did not show any valuable reactivation potency, only their applicability in different areas (Petronilho et al. 2015). Hydroxylamines by Lo and Ganguly, together with some novel structures presented by Bhattacharjee et al, showed some promising possibilities; however, their results need to be supported by in vivo biological data (Bhattacharjee et al. 2012; Lo and Ganguly 2014). Non-oxime based reactivators published by Katz and co-workers have already been supported by validating their potential in vivo (Katz et al. 2015; Bierwisch et al. 2016). Amodiaquine acts as broad-spectrum reactivator of OP; however, it has a high inhibitory potency towards hAChE limiting its potential use. Based on these results, amodiaquine has become a valuable template in order to find alternatives for oxime based reactivators (Bierwisch et al. 2016; Cadieux et al. 2016).

Improvement of oxime dissociation to the active oximate anion is a very important issue which scientists must bear in mind in order to develop highly efficient reactivators. Formation of oximate anion is essential to confer the compound reactivation properties. Dissociated and undissociated forms of reactivator with the pK_a 7.40 have equal concentration at pH 7.40. With pK_a 7.00, relative concentration of oximate anion occurs in about 71 % and it is around 10 % at pK_a 8.35 within physiological conditions. These data suggests the optimal pK_a range laying between 7.00 and 8.35 for efficient reactivation where at least 10 % of oximate anion occurs (Musil et al. 2016). The pK_a value is also important indicator for penetration through biological membranes especially through BBB. Charged (mono- and bis-quaternary) reactivators have only very limited permeability, even though this still seems be enough to provide effective concentrations to restore activity of brain AChE. Furthermore, BBB permeation of the compound is even more limited when reactivator is dissociated and oximate anion is formed. The pK_a values of brain effective drugs were found ranging between 7.5 and 10.5 (Pajouhesh and Lenz 2005). In order to ensure proper pK_a value of the novel reactivators, some modifications of the standard aldoxime moiety have been made to meet this criterion. However, none of the functional groups presented by 2-hydroxyiminoacetamides (Sit et al. 2011; Kovarik et al. 2013; McHardy et al. 2014; Karade et al. 2014), amidoximes (Saint-André et al. 2011; Kliachyna et al. 2014), hydroxamic acid (Saint-André et al. 2011), keto-oximes (de Koning et al. 2011b; McHardy et al. 2014) and amidine-oxime (Kalisiak et al. 2011, 2012) led to satisfactory results obtaining more efficient reactivators. Mostly, all these reactivators have comparable or lower reactivation ability to 2-PAM.

It has been reported that VX-agent is predominantly acting on the peripheral AChE, while the more lipophilic G compounds are primarily centrally acting nerve agents (Bajgar et al. 2007b). Mostly, the effects of nerve agents vary in different brain areas from relatively resistant striatum to very sensitive structures such as ponto-medullar area and frontal cortex (Bajgar et al. 2007a). Reactivation of peripheral AChE is significant for neuromuscular transmission important for proper ventilation. Similarly, maintaining the activity of AChE in the central respiratory control centre in the ponto-medullar area is considered to be vital for survival (Goswamy et al. 1994; Sungur and Güven 2001; Bajgar et al. 2007b). The evidence for presence of charged reactivators in the brain have already been demonstrated (Falb and Erdmann 1969; Cassel et al. 1997; Sakurada et al. 2003). However, the penetration to the BBB depends on the administered doses and on the targeted brain area (Bajgar et al. 2007a, b). In general, concentration 10^{-4} to 10^{-5} M achieved in the brain should be sufficient for reactivation of the central AChE, especially in ponto-medullar area resulting in the minimal level of AChE activity (about 5–20 %) for the survival (Bajgar et al. 2007a). This fact also reflects the importance of the sufficient dose to be administered in order to ensure central AChE activity. Uncharged reactivators should be the solution when they could be administered in a lower dose necessary to reach ponto-medullar area. Furthermore, enhanced lipophilicity of such compounds help reactivators to reach different brain regions and more efficiently restore the central AChE function and by that avoiding belated neurological symptoms (dos Santos et al. 2016). Thus, the developed uncharged reactivators are mainly based on dual binding site strategy in order to increase CNS bioavailability for the OP-inhibited AChE. However, it must be pointed that such lipophilic compounds may be also hampered with increased toxicity especially in the CNS.

AChE dual binding site strategy received particular attention in the mid-nineties. Pang et al. proposed computational study supported by the synthesis and in vitro evaluation of the FDA approved AChE inhibitor 9-amino-1,2,3,4-tetrahydroacridine (THA) used for palliative treatment of AD. Their outcome significantly approved benefit of dual site binding strategy. Specifically, connection of the two THA molecules through the alkylene chain yielded in compound which was 1000 times more potent and 10,000 times more selective in inhibiting rat brain AChE over the THA alone. Optimal length of the alkylene chain to reach each of the active site was determined to be 7 methylene groups between THA cores (Pang et al. 1996). Such approach postulated that targeting both anionic sites of AChE resulted in more potent AChE inhibitors. Following this idea in the research of AChE reactivators, PSLs acting as anchor of AChE enhance reactivation potency of CAS ligands (de

Koning et al. 2011b). In this context PIQ (Mercey et al. 2011), tryptoline (Renou et al. 2014) and tacrine are largely exploited scaffolds as PSLs (Kliachyna et al. 2014; Nepovimova et al. 2016). Such dual binding reactivators brought interesting in vitro reactivation ability comparable to the bis-quaternary standards. However, in vivo assessment pointed to poor solubility leading to low bioavailability of the compounds (Calas et al. 2016). Also the different routes of their administration do not provide unambiguous answer whether the dual binding strategy for AChE reactivators is reasonable to follow (Sit et al. 2011; Radić et al. 2012; Kovarik et al. 2013; Wei et al. 2014). Taken together, AChE dual binding reactivators are under development and on the uncertain way to replace currently approved charged reactivators. Trade-off between increased BBB penetration and maintenance of desired solubility for in vivo administration might further tentatively favour mono-charged reactivators, especially with the combination of suitable PSL. Mono-quaternary compounds with PSL may establish future leading trail in the effort of replacement bis-quartenary compounds.

Conclusion

Over the 60 years of extensive research, many efforts have been devoted to the development of new reactivators which would eliminate three major drawbacks of currently available antidotes. Although the most efficient reactivators clinically used are mono- or bis-pyridinium aldoximes, recent research slightly digresses from this dogma and presents a new variety of structures offering new possibilities. To date, the dogma of oxime functional group as the nucleophile has not been overcome by new chemical entities available for potent in vivo reactivation (Lo and Ganguly 2014; Bhattacharjee et al. 2015; Katz et al. 2015). The solution of three main drawbacks has become major point of interest.

1. Dealing with broad-spectrum reactivator would rather be attainable with difficulties due the fact that every diversity in molecule of inhibitor needs a specific structure of the reactivator (Antonijevic and Stojiljkovic 2007; Jokanović 2012).
2. Increased BBB permeation is often one of the main issues discussed in many articles, but the efficient CNS reactivation in vivo has not been supported to date by the literature data (Korabecny et al. 2014). Moreover, increased BBB permeation may lead to the increased CNS toxicity of reactivators.
3. The research dealing with the "aged"-AChE drawback has demonstrated the possibility of its elimination on the computational level (Topczewski and Quinn

2013). However, this hypothesis needs direct proof of concept within in vitro/in vivo techniques.

Currently developed compounds exceed in some aspects the reactivation profile of known reactivators. The obtained results are often insufficient with none or incomplete in vivo study and/or with missing pharmacokinetic parameters. The results are also hampered by difficulty of different experimental conditions which may result in huge data variations (Sterner et al. 2013; Worek and Thiermann 2013). On the other hand, the experimental data shows that some compounds might be equivalent to standard oximes that are currently available. To conclude, combinations of CAS and PAS ligands may foster development of new and potent reactivators, favouring monocharged compounds.

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