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LETTERS

Role of Acetylcholinesterase (AChE) reactivators in the treatment of Organophosphorus poisoning: *in vivo*, *in vitro,* and *in silico* studies

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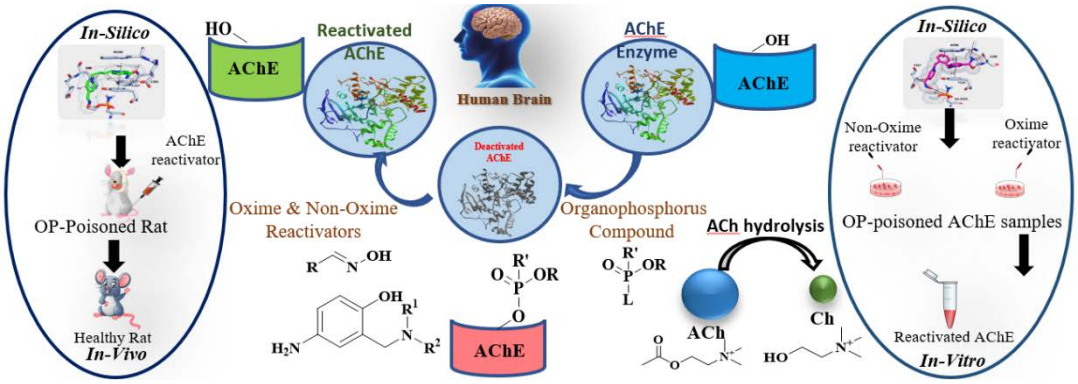
Review

ABSTRACT



Chemical warfare agents (CWAs), especially organophosphorus (OP) compounds, are known for their extreme toxicity causing inhibition of acetylcholinesterase (AChE) enzyme activity due to covalent phosphorylation. This leads to the functional impairment of muscarinic nicotinic acetylcholine receptors, resulting in severe ill effects that ultimately lead to death. For OP poisoning, AChE reactivators play a crucial role in the treatment process. Among several AChE reactivators, oxime reactivators are mainly employed for treating OP intoxication. Nevertheless, these are associated with certain drawbacks, such as their toxic effects, low blood-brain barrier (BBB) penetration, less reactivation in the central nervous system (CNS), and inefficiency towards all nerve agents, and AChE. As a result, new therapeutic strategies are required. Recent attempts are focused on the design and synthesis of uncharged oxime or non-oxime reactivators which can overcome the limitations of oxime-based reactivators. A novel class of non-oxime reactivators is gaining interest, including compounds like Mannich phenols, chloroquine, and some general bases. This review is a novel attempt to incorporate various possible oxime and non-oxime AChE reactivators for OP intoxication along with their *in vitro, in vivo*, and *in silico* studies.

Keywords: Organophosphorus; Oximes; Non-oximes; AChE; Reactivation

INTRODUCTION

Organophosphorus compounds (OPs), termed as nerve agents, belong to the organic class of phosphorus-based moiety and possess a wide range of domestic and industrial uses.[1] They have been employed as warfare agents due to their non-selective toxicity in wars.[2] These include terrorist attacks against civilians (Matsumoto, Japan in 1994 and Tokyo in 1995)[3] and the most recent civil war in Syria. All of them which were developed as chemical warfare agents have been prove to be highly toxic and dangerous to humans.[4,5] Before being utilized as weapons during warfare, OPs were first developed and used on a large scale as insecticides[6] in developing countries during the 1930s. Despite their use as insecticides, these are very toxic with higher rates of contamination. OP poisoning can take place through the skin, oral contact or respiratory tract. These inhibit the action of the acetylcholinesterase (AChE) enzyme, accountable for the hydrolysis of the neurotransmitter Acetylcholine (ACh) and if no immediate treatment is given, they may result in prolonged inhibition.[7]

OP poisoning has been a leading cause of hospitalization and ICU admissions of patients.[8] On an average, around 30,00,000 acute OP intoxications occur every year, of which 3,00,000 result in fatalities.[9] OPs, in particular, affect the muscle responsible for breathing, eventually leading to death.[7,10,11] For hydrolysis of neurotransmitter, ACh, at the cholinergic synaptic site[12], AChE is a very important enzyme that belongs to the alpha/beta hydrolase family generally found in the central and peripheral nervous system.[13] Two binding sites are present in AChE, i.e., active and peripheral anionic sites.[14–19] The primary biological function of AChE is the rapid hydrolysis of the cationic neurotransmitter, ACh, at neuromuscular junctions and neuronal

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synapses[20,21] into choline and acetic acid catalyzed by the active site of AChE. Nerve gas agents are the most common AChE inhibitors (Figure 1), which mainly contain OPs that bind to the -OH of serine residues and block the action of AChE in the hydrolysis of ACh into acetic acid and choline.[22–24] This inhibition of AChE action leads to the accumulation of ACh at the neuron synapse[25], resulting in several symptoms of poisoning, such as seizures and respiratory arrest.

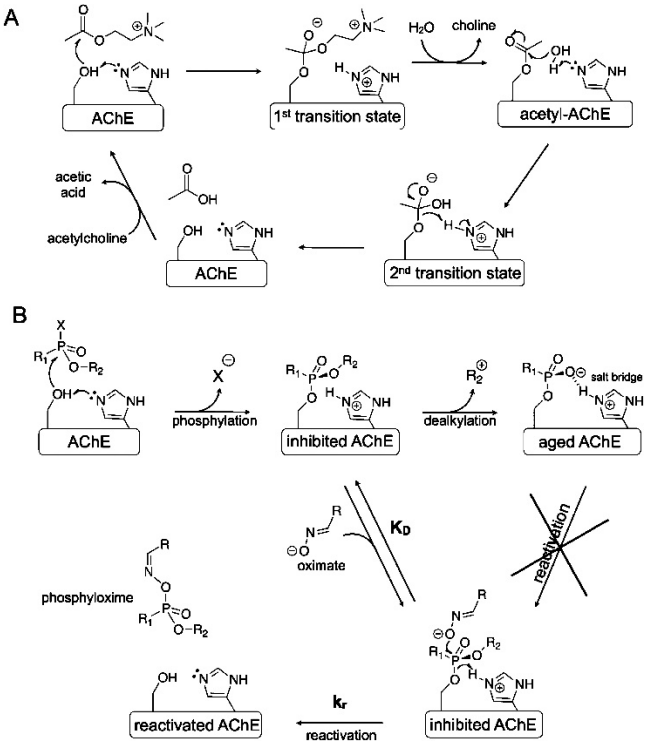


Figure 1. Mechanism of (A) acetylcholine hydrolysis by AChE. (B) AChE inhibition by organophosphorus nerve agents, aging, and reactivation by oximes. Reprinted with permission from Mercey, G. et al. Copyright (2012) American Chemical Society.

The inhibition occurs via hydrolysis of the serine residue[14–19] by the active site of the enzyme AChE. The overstimulation of cholinergic receptors occurs due to this AChE inhibition which causes neurotransmitter accumulation[22] into central and peripheral cholinergic sites. Excessive salivation, lacrimation, and neuromuscular block are major intoxication-related symptoms.[23] Several chemical events are involved in the inhibition of AChE by OP.

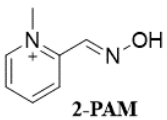
When the serine residue of AChE performs a nucleophilic attack on ACh, an unstable tetrahedral intermediate is formed, which breaks down into choline and acetyl enzymes. Histidine residue activates a water molecule and attacks the acetyl enzyme, resulting in AChE regeneration through some other transition states.[26] The choline released during hydrolysis is adsorbed and used as a substrate for ACh–resynthesis.[27] The inhibition is caused by serine phosphorylation at the enzyme active site. The attack of nucleophilic serine on the electrophilic P-atom results in the formation of a bipyramidal intermediate, and the cleavage of the leaving group results in the formation of the phosphorylated enzyme. Its spontaneous recovery rate is extremely slow.[28]

Researchers have developed several compounds to overcome problems such as low BBB penetration and lipophilicity in recent years. This article compares numerous oxime and non-oxime AChE reactivators based on *in vivo*, *in vitro*, and *in silico* studies. This review aims to compare AChE reactivators and find the most appropriate molecules to treat OP intoxication.

AChE Reactivators

Oxime Based

Cholinesterase reactivators are commonly used as a part of the antidote treatment against nerve agent poisoning caused by pesticides.[29] They carry out the reactivation of human acetylcholinesterase (hAChE) inhibited by OP compounds by disrupting the inhibitor-enzyme bond.[30] Oxime-based cholinesterase reactivators such as pralidoxime (2-PAM), oxime trimedoxime, obidoxime, and other agents including anti-cholinergic (ex-atropine) and anticonvulsants (ex-benzodiazepine) have been used as antidotes for OP intoxication.[30–35] An oxime is responsible for the reactivation of the OP-inhibited enzyme through the nucleophilic displacement of the OP moiety attached to the catalytic serine of the cholinesterase (ChE) active site.[31] Numerous mono- and bis-pyridinium oximes with quaternary nitrogen atoms do not cross the blood-brain barrier (BBB) efficiently due to their permanent positive charge[36] and thus do not reactivate brain AChE. As a result, several research groups are recently emphasizing the development of a new generation of AChE reactivators which include uncharged molecules containing protonatable groups.[21,37] Various heteroatoms, aromatic rings, and double bonds were added to the linkage in order to strengthen various enzyme inhibitors interactions such as π-π interactions and hydrogen bonding within the AChE binding pocket.[38] The molecules developed over the last 70 years have serious drawbacks, including their failure to cross the BBB, limiting their potential to reactivate AChE in the CNS, adequate toxicity, and a lack of broad-spectrum activity.[36,39]

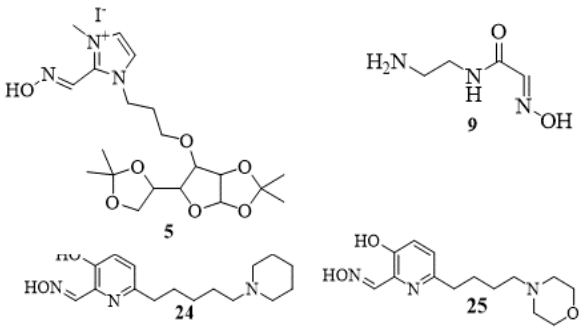


2-PAM

Pyridinium oxime reactivators of phosphorylated AChE such as antimuscarinics, anticonvulsants, 2-PAM, trimedoxime, methoxime are used to treat nerve agents.[40] Pyridinium oximes displace the phosphoryl group from serine, the active site of AChE,[41] due to their considerable nucleophilicity, but they have a major limitation in terms of bioavailability in the CNS despite 60 years of research.[41,42] The positive charge of pyridinium oximes prevents them from passing through the BBB, resulting in significant CNS reactivation.[43–45] In spite of years of research, no oximes have been identified as broad-spectrum reactivators for a variety of OP.[46] The reactivation of the aged conjugates is contrary to known antidotes.[47,48] Commercially available

antidotes for CWAs include 2-PAM and obidoxime.[49] Cyclosarin and Russian-VX are less effective against soman as compared to 2-PAM and obidoxime.[50,51] Despite its strong therapeutic effects, atropine cannot alleviate OP-induced ill effects at nicotinic receptors in the brain.[52] Some BBB permeable oximes have been reported in the literature, but nothing has been synthesized that can act as AChE reactivators due to low functionality and considerable toxicity.[36] Although DAM and MINA demonstrated substantially lesser reactivation in cholinesterase when compared to 2-PAM and other quaternary oximes, they were found to show better penetration ability.[36,53,54] Pro-drugs which undergo oxidation *in vivo* in the CNS to form active quaternary oximes were used as an alternative to this problem.[55]

Although hundreds of compounds exist, some of them (**1**-**58**) (SI Table 1) were chosen and studied based on their ability to overcome these drawbacks. Each compound was discovered to have unique properties; some were lipophilic, while others had higher permeability. Various *in vivo* or *in silico* studies, such as docking and reactivation, were performed in order to obtain a compound of perfect choice (SI Table 5, 8 and 9). VX-hAChE was found to be 8 times more revived by oximes **1**-**3** than by pralidoxime, but tabun-hAChE was not readily reactivated by oximes **2**-**4**.[56] K203 was found to be more efficient against rat enzyme.[57] Oxime **24** and **25** were found to be the most potent due to their broad spectrum of reactivation and are reported to cross the BBB efficiently *in vitro*, resulting in enhanced therapeutic decontamination for OP exposure.[58] These studies provide insight into the use of oximes to treat OP exposure and their potential against various aspects such as penetrating the BBB. These oximes are listed in SI Table 1.



The standard OP treatment for AChE inhibition includes HI-6, obidoxime, and 2-PAM, all of which share a pyridinium aldoxime group. However, these oxime reactivators have some drawbacks, including low BBB penetration, adequate toxicity, and a lack of broad-spectrum activity, implying that an oxime is insufficient to counteract all nerve agents. Although a few uncharged oximes (MINA, DAM, and others) capable of BBB penetration demonstrated lower reactivation and significant toxicity compared to other quaternary oximes. As a result, newer alternatives have been taken into account in order to combat OP toxicity.

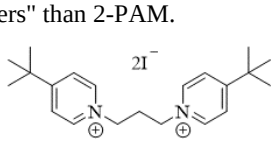
Non-Oxime Based

During the last few decades, *in silico* pharmacophore has gained prominence in identifying novel drugs. Pharmacophores are a set of steric and electronic characteristics required for optimum interactions with a particular receptor in order to counteract its biological activity. Initially, Bhattacharjee et al. reported five novel non-oxime reactivators from a database that demonstrated tenfold efficacy compared to 2-PAM against DFP-inhibited AChE, based on *in silico* pharmacophore modeling and virtual screening.[50] Based on reactivation capability, the first 67 compounds were shortlisted out of 2,90,000 compounds using the WRAIR-CIS database. Based on *in silico* investigations for BBB penetration, water-octanol partition (ClogP), oral LD50 toxicity, and OP active-site binding affinity suppressing AChE, a further 10 non-oximes (**59**-**68**) were shortlisted. These non-oximes are shown in SI Table 2.

In an *in vitro* DFP-inhibited electric Eel AChE, the K_r values of these non-oximes were found to be 10-fold of 2-PAM. They also exhibited substantial efficacy against 2-PAM toxicity in *in vivo* investigations. *In silico* assessments of drug-like features and tolerable toxicity patterns revealed that compound **60** had superior *in vitro* efficacy than 2-PAM (SI Table-6), while the remaining nine were within 10-fold of 2-PAM. A number of methods were studied to compare and conclude the best possible AChE reactivator against OP poisoning.

Bispyridinium Non-Oxime Based

These are nicotinic antagonists which have been found as potential oxime reactivator substitutes. The removal of the reactivating CH=NOH group of oxime results in the synthesis of non-oxime bis-pyridinium compounds, which still exhibit inhibitory activity via a negative allosteric mechanism.[59] In 2017, Scheffel C and coworkers studied that different substituted groups at different positions of the chemical structure contribute to different reactivation potency.[60] The inhibitory activity is dependent on the length of the alkane linker between two pyridinium moieties. Currently studied pyridinium non-oximes include MB327, MB408, MB442, MB444, MB505, MB782, MB454, MB414, and MB266[61], which showed some potential to reactivate OP-inhibited AChE. Structures of such bispyridinium non-oximes (**69**-**80**) are shown in SI Table-3. Compound **70**, MB327 [1,1′-(propane-1,3-diyl) bis(4-tert-butylpyridinium) diiodide][62], was discovered among these compounds during tests with Soman poisoned human intercostal muscles. It was found to be capable of restoring neuromuscular function. Moreover, *in vitro* muscle force examinations and solid supported membrane (SSM)-based electrophysiology of soman-poisoned rats were used to investigate MB327 and its regioisomers, namely, PTM0001 and PTM0002.[63] SSM-based electrophysiology studies revealed that MB327 (**70**), as well as its regioisomers, are better "Re-sensitizers" than 2-PAM.



70

Similarly, non-oxime compounds such as MB408, MB442, and MB444 demonstrated better therapeutic potential against OP nerve agents when combined with oxime reactivators and

atropine.[64] Sarin LD50 data for MB444 showed that it was more effective than MB408. All of these MB compounds increase animal survival time and thus survival rate. As a result, these bispyridinium non-oximes are better alternatives to oxime reactivators as they can overcome some of their limitations and exhibit improved reactivation and inhibition potency against OP-inhibited AChE.

BIOLOGICAL STUDIES

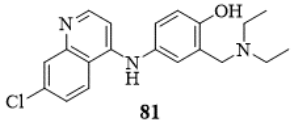
REACTIVATION STUDIES

The reactivation of OP-inhibited AChE is the basic mode of action of oximes. Direct pharmacological effects of the oxime have been the subject of speculation. Renou et al. developed four Donepezil-based compounds in a study published in 2016. Since Donepezil was found to inhibit the activity of AChE, its derivatives were subsequently tested for their reactivation activity. As shown in SI Table 1, oximes **1-3** had an affinity for reactivating VX-inhibited human AChE that was about 8-fold higher than pralidoxime. Oxime **2** (SI Table 1) had a 5 to 11-fold higher affinity than HI-6 and pralidoxime. The reactivity and affinity of an oxime toward OP-inhibited AChE is used to determine its reactivation capacity, which can be evaluated *in-vitro/in-vivo* using dissociation and reactivity constants. Half maximal inhibitory concentration (IC50) is the most commonly used and informative measure of drug efficacy, indicating how much drug is required to inhibit a biological process by half. Compound **1** (SI Table 1) was discovered to be a mild inhibitor with an IC50 value of 1.0±0.1 µM. The indanone moiety possessing carbonyl functionality boosts binding affinity for VX-hAChE but lowers the rate constant, as seen in the results for compounds **1** and **2** (SI Table 1).[36] In 2020, Sharma et al.[55] demonstrated that charged imidazole–aldoximes have higher reactivation activity than the uncharged and developed compounds **5-7** (SI Table 1). The lower log D values suggested a greater lipophilic nature when compared to the standard oxime used as a reference. Compared to butane and pentane linkers, lower K_a values for compound **5** demonstrated an excellent affinity for OP-AChE adducts with propane linkers. K203 was discovered to be the most potent reactivator, with a second-order rate constant of 2142/minM, which was 51 times higher than obidoxime (42/minM). It has two quaternary pyridinium rings with four-carbon connections between them, which could be the cause for its higher efficacy based on the structure-activity relationship (SAR). Overall, K203 reactivated 60% of cholinesterase activity at humanly attainable concentrations. It also interacted better with the human enzyme HssAChE and tabun-inhibited AChE, which was favored by overall second-order kinetics. In contrast, both oxime and pralidoxime failed to reactivate tabun-inhibited AChE.[53] Both AChE and BuChE possessed a higher affinity for dichloride-substituted oximes over monochlorinated oximes. This demonstrates that the extra chlorine atom interacts with active site residues and helps to stabilize the oxime-enzyme complex.[58] The reactivation of reactive OP is based on the chemical degradation of OP, but their limited potential is due to a lack of activity in AChE reactivation. In 2021, Cannon et al.[56] developed a nine-membered oxime library (compounds **9-18**, SI Table 1), with compound **9** serving as the lead structure due to its excellent OP reactivity. The amine-terminated scaffold is also hydrophilic, which helps in preventing the negative percutaneous absorption linked with lipophilic DAM. Enzyme studies using human as well as electric Eel subtypes of OP-inactivated AChE revealed that they were susceptible throughout a broad spectrum. They were able to retain reactivation in paraoxon-inactivated AChE by up to an 8-fold relative rate increase in controlled conditions compared to DAM. K131, K142, and K153 (SI Table 1) could not reactivate paraoxon-inhibited HssAChE successfully.[50]

The reactivation of oximes was described to follow a two-step nucleophilic mechanism, and the same mechanism was later chosen for the reaction of non-oximes with OP-inhibited AChE. It has been claimed that the dissociation constant (K_d) quantifies the reactivation process and is inversely related to the affinity of the non-oxime reactivator to OP-inhibited AChE. The reactivation rate constant (K_r) physically signifies its potential to remove the OP residue from the AChE molecule. In the case of complete enzyme reactivation and [NOX]>>[EP], a pseudo-first-order rate equation can be derived.[50]

$$k_{obs} = \frac{k_r * [NOX]}{K_D + [NOX]}$$

According to MC de Koning et al. (2018), **109** (SI Table 12) has a higher reactivation potency than 4-amino-2-diethylaminomethyl phenol (ADOC). In this regard, he investigated the *in vitro* reactivation kinetics of compound **109,** the structural analogues of ADOC, with human AChE inhibited by sarin, VX, cyclosarin, and paraoxon. In addition, the reactivation potency of **109** and ADOC (**82**) in human and guinea pig OP-inhibited AChE was compared.[65] Human or guinea pig erythrocyte ghosts were incubated for 15 minutes at 37 °C with small volumes (1% v/v) of sarin, cyclosarin, VX, or paraoxon to achieve an AChE inhibition of ≥95% of control activity. Dialyzes of inhibited and control samples were performed overnight at 4 °C (phosphate buffer, 0.1 M, pH 7.4). Then, for time intervals ranging from 1 to 60 minutes, non-oxime compounds were added, and their reactivation kinetic parameters were recorded. The results revealed that compound **82** had a low reactivation affinity (K_d >430um). It demonstrated a reduced reactivation rate with sarin and paraoxon inhibited AChE but a relatively high reactivation rate with VX-inhibited AChE. Compounds **101** and **102** (SI Table 12), having separation of methyl groups, possessed lower affinity and reactivity, so they were only partially able to reactivate OP-inhibited AChE. However, compound **109** proved to be the most successful in reactivating all tested OP-AChE combinations.



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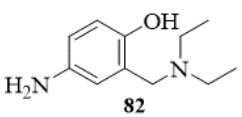


82

In addition to ADQ, another antimalarial drug, Chloroquine, which lacks the phenol entity, demonstrated reactivation capability. The 9-chloroquine ring caters to this ability. SI Table 4 (**81-86**) depicts the structure of Chloroquine and its derivatives possessing reactivation ability.

In Vivo/In Vitro Studies

The word "vivo" means "inside the alive". It is a collection of investigations, methods, and tests carried out on a living animal, such as a lab animal, whereas “vitro” indicates "without living organisms". *In vitro* tests are conducted without the use of a biological creature. These studies provide useful information about reactivation and medication efficacy. Renou et al.[36] investigated compound **1** (SI Table 1) for its ability to reactivate sarin-inhibited human AChE *in vitro*. It was discovered to be less efficient than HI-6 due to a lower affinity for sarin–hAChE and a lower reactivation rate constant. When oximes **2-4** (SI Table 1) were evaluated for *in vitro* reactivations of tabun and paraoxon-inhibited human AChE, they showed only negligible activity on tabun-hAChE. However, they were found to be highly active on paraoxon-hAChE, being more efficient than HI-6, despite the latter being known to be a poor pesticide reactivator. Oximes **2** and **3** had similar reactivation characteristics despite their structural differences.[36]

The glycosylated imidazolium aldoximes synthesized by Sharma et al. (2020)[55] were tested for *in vitro* biological activity (compound **5-7**, SI Table 1) using reactivation studies. Various parameters, including the orientation of the nucleophilic oxime with respect to the phosphate group in the OP-AChE adduct, its potential, and age influence the reactivation of OP-inhibited AChE. Kinetic studies of reactivation were used to test the *in vitro* activity of compounds **5-7**. *In vitro* reactivation tests were performed on the synthesized oximes against ethylparaoxon and methyl paraoxon-inhibited electric Eel AChE. Quaternary uncharged oximes regenerated inhibited Eel AChE to an extent of 27-40% and had lower pk_a values and improved reactivation of charged aldoximes **(5-7)**. The K_d values of all oximes were much more significant than those of pralidoxime and obidoxime. In comparison to pralidoxime, oximes **17** and **18** (k_r: 0.096 min^{-1} and 0.107 min^{-1}, respectively) have a greater reactivity rate constant (0.082 min^{-1}). Only oxime **18** (0.107 min^{-1}) had a rate constant that was equivalent to obidoxime (0.111 min^{-1}) in the instance of ethyl paraoxon (SI Table 5). In the instance of methyl paraoxon, no oxime exceeded the reactivity rate of the tested reference oxime. In both *in vivo* and *in vitro* rodent investigations, K203 came out to be the most effective reactivator of tabun-inhibited AChE. The effectiveness of K203 on the human brain AChE was first discovered in a rodent study. K203 reactivated more than 60% of tabun AChE and was more effective than obidoxime. When the overall rate constants of human and rat enzymes are compared, K203 decreases only 7.5 times while obidoxime decreases 149 times. According to quantum studies, K203 and obidoxime are very effective in reactivating inhibited MmAChE. It is discovered to be most reactive in rat AChE and not in human AChE. Out of VX, sarin, cyclosarin, and paraoxon, oxime 25 recovers >80% of total cholinesterase activity in ~10 minutes, with the exception of cyclosarin, which takes 60 minutes.[53] *In vitro* tests for 9 compounds (compounds **9-17**, SI Table 1) were carried out with a focus on dual action. They demonstrated the exceptional potential of POX in skin decontamination *in vitro*. These oximes were developed with a pH of 10.5, and their efficiency was tested *in vitro* using POX decontamination on porcine skin. Compounds **12-14** were more efficient than DAM compounds in the POX penetration blocking. These findings indicate a good relationship between skin decontamination efficacy and chemical reactivity in solution. Similarly, *in vitro* studies, PAMPA assay showed a passive transport across the BBB for compounds **12** and **15**. *In vitro* studies also show that compounds **28-30** do not reactivate HssAChE or HssBChE.[56] *In vivo* studies, compounds **31-36** (SI Table 1) have been shown to be effective against CNS activity in nerve agent exposure.[58] Former K-oxime compounds K048 and K074 were used to identify 15 new AChE reactivators. Their molecular framework was developed by heteroarenium moiety. They were compared to commercial HssAChE and tested on tabun, paraoxon, and methyl paraoxon-inhibited human AChE. A few chemicals showed promising behavior in their capacity to reactivate HssAChE.[66] JR595 (**52**) was identified as a highly metabolically stable oxime for 1321N1 in human liver microsomes, monocytotoxic oxime for trocyma cell lines, and SH-SY5Y neuroblastoma from *in vitro* experiments. After the intramuscular injection in mice, its pharmacokinetic profile was studied. It got absorbed immediately into the blood after 15 minutes, with a simultaneous supply to the brain at ~40% of its blood content. *In vitro* investigations also revealed that the reactivation parameter of human and mouse AChE inhibited by VX and sarin did not differ significantly.[67] In 2018, Zorbaz et al.[58] developed a 6-chlorinated bispyridinium mono oxime that successfully reactivated human-AChE that had been inhibited by nerve agents. The complete reactivation of the dichlorinated analogue of K027 was 3, 7 and 8 times higher than K027 for Sarin, cyclosarin and VX-inhibited AChE respectively.

Computational Studies

Molecular Docking Studies

Docking studies comprise of theoretical investigations of how two or more structures fit together and interact. The docking study of K203 revealed that it interacts more with the human enzyme HssAChE, having intermolecular interaction energy equal to -101.94 kcal/mol. H-bonding experiments with the two amino acid residues Thr 238, Gly 234, and two water molecules K203 indicate the stability of MmAChE.[53] According to docking models of VX-inhibited mouse AChE, both **25** and **26** (SI Table 1) are coupled with 3-hydroxy-2-pyridine aldoxime moiety in an elongated conformation with the active site and establish a π-π interaction at the site of choline-binding with Trp86. Despite being close to the appropriate O-P distance (4-5 Å) for nucleophilic attack on P=O group, both oximes **25** and **26** do not acquire the proper orientation of the oxime group. Better stabilization of oxime **26** in productive conformation (the only difference between oxime **25** and **26** is the morpholine ring

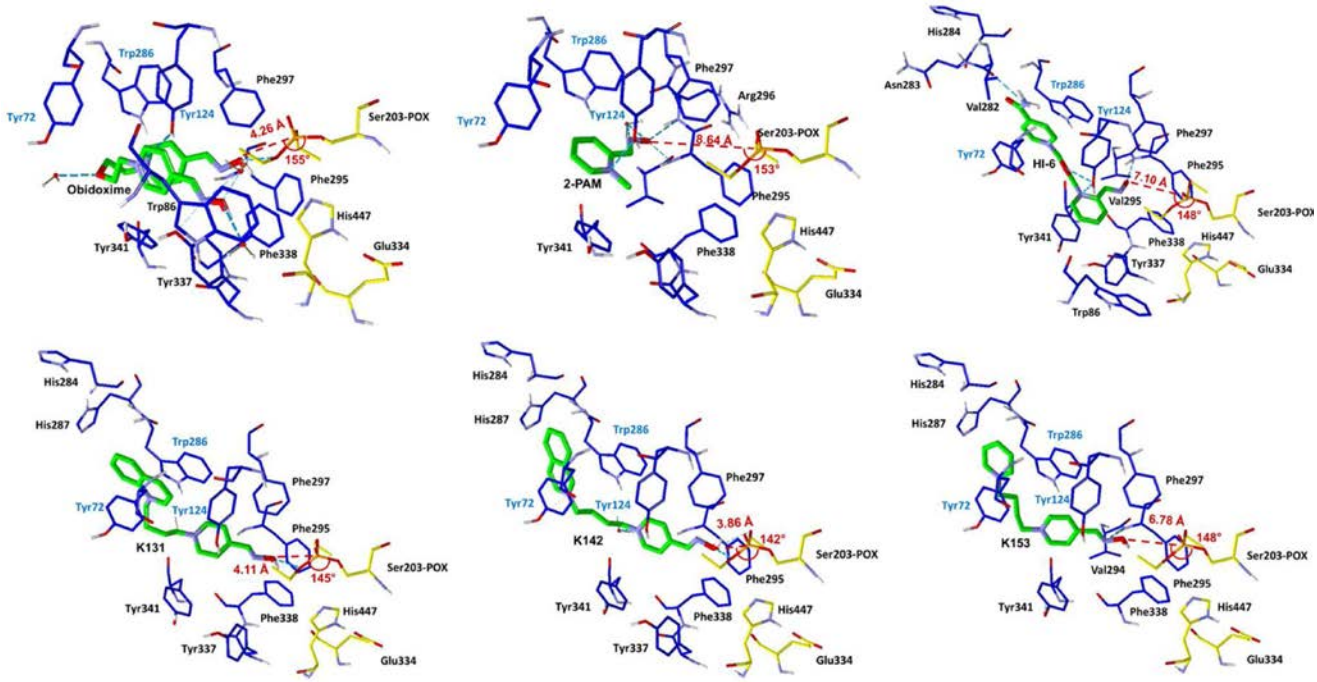


Figure 2: Best poses of the oximes inside the complex *Hss*AChE/POX. Distances $P_{OP} - O_{Ser203}$ and angles $O_{ox} - P_{OP} - O_{Ser203}$ are shown in red[57]

orientation) could lead to oxime **26** having a better reactivation efficiency (in terms of k_1) than **25**. In order to investigate the binding mechanism of **25**, molecular modeling of the hBChE complex with oxime **25** was carried out, and the maximum reactivation efficiency in terms of k_2 was shown. Oximes **(24-27)** inhibited by 5 OPs (VX, sarin, cyclosarin, paraoxon, and tabun) were tested as reactivators, and the majority were observed to get reactivated OP-inhibited AChE to a high extent, with 80-100% enzyme activity, compared to HI-6 (standard reactivator). Because of the higher value of k_2, all of the **24-27** had the best molecular weight (280-400), the best number of H-acceptors (5-7), and the best lipophilicity. All **24-27** were found to be passively transported over the BBB in a parallel artificial membrane permeation study, however, HI-6 and 2-PAM were found to be impermeable in a PAMPA assay.[54] None of the synthesized oximes were able to reactivate paraoxon-inhibited cholinesterase satisfactorily, according to docking studies for **28-30**.[57] The results of molecular docking confirmed the *in vitro* findings of significant hAChE inhibition by symmetrical compounds (K298, K344, K474, K524). The structure-activity relationship also revealed that structurally related compounds have stronger inhibitor effectiveness against hAChE. Time-dependent concentration changes in rat plasma of K298 and K524 were examined to determine an approximate time for prophylactics administration. Maximum plasma concentrations of 100nM were attained in 30 minutes for K298 and 39 minutes for K524. The *in vivo* prophylactic effect of oximes was also investigated. None of the inhibitors could reduce acute soman-induced toxicity in mice. Only K474 increased the antidotal treatment slightly.[31] Compounds **31-36** (SI Table 1) may be used to treat nerve agent poisoning by inhibiting CNS activity.[58] Docking studies were also used for a better understanding of *in vitro* results, and some newly developed compounds were discovered to outperform the reactivation for POX-inhibited HssAChE of previously known compounds.[66] Different reactivation molecular docking studies were performed to predict binding modes, and the results agreed with experimentally calculated values of all kinetic parameters (Interaction shown in Figures 2 and 3).[57]

The binding energies of all the compounds at the active site of the structure of tabun-inhibited AChE were calculated to gain a better knowledge of the therapeutic properties, using Chem Navigator's 3-D protein-ligand (3DPL) docking protocol.[79] This docking protocol made use of the 2GYU protein structure. The binding energies of the compounds for normal as well as OP-inhibited AChE were calculated in order to investigate their reactivation efficacy and competing inhibitory activity at all possible sites. 3DPL is primarily concerned with steric, H-bonding, hydrophobic, and electrostatic interactions. Hence, 3DPL docking calculations were performed for the newly discovered non-oximes in order to test their ability to bind to the OP-inhibited AChE crystal structure (SI Table 6). From the difference in binding energy between AChE active sites, it is clear that non-oximes **65** and **67** possess a higher affinity for OP-inhibited AChE, while others show a higher affinity for normal AChE. Compound **63** has the same affinity for both active sites. This provides insight into the interaction and efficacy of newly discovered non-oximes at the active site of OP-inhibited AChE.

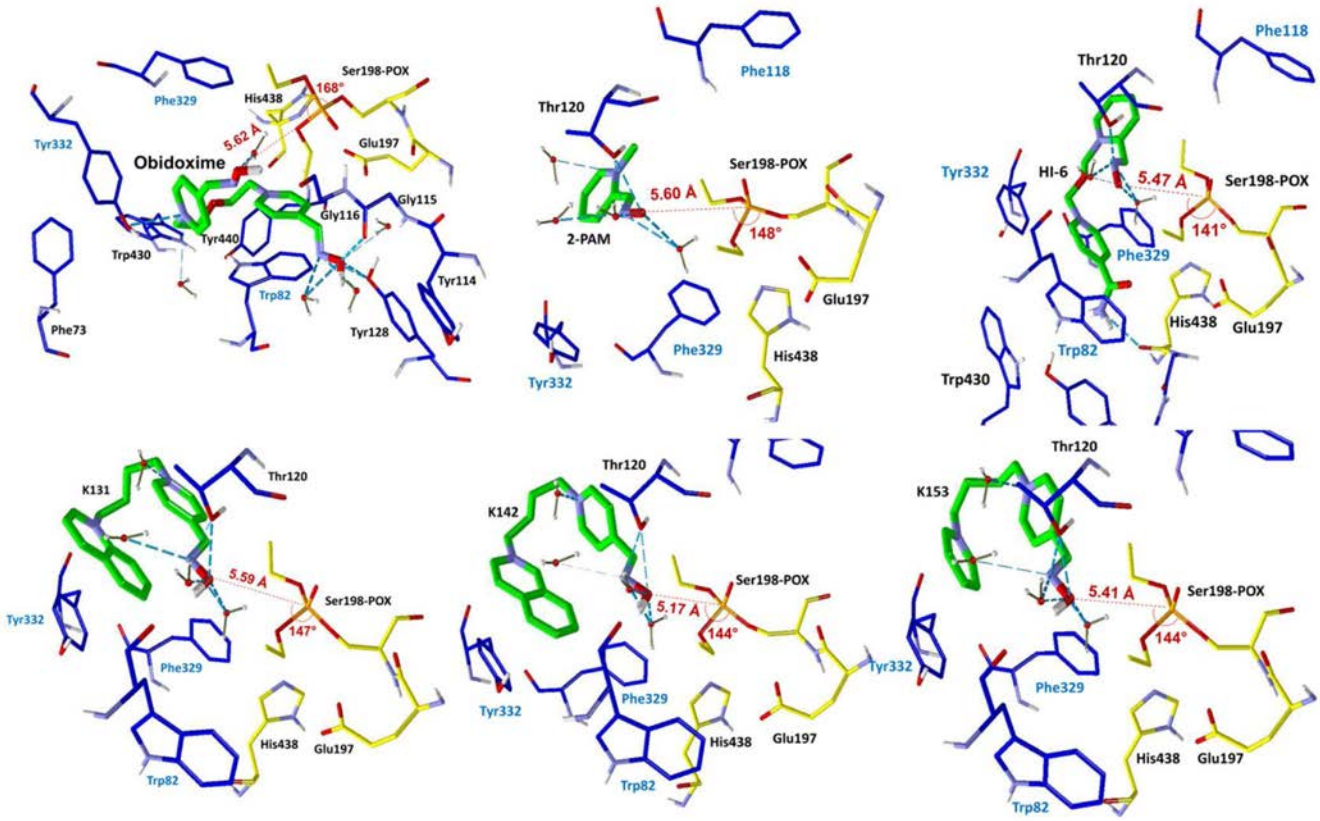


Figure 3: Best poses of the oximes inside the complex HssBChE/POX. Distances POP − O_{Ser203} and angles Oox − POP − O_{Ser203} are shown in red[57]

In Silico Studies

The oximes were examined for their molecular properties with *in silico* studies using their pk_a values to evaluate their potential to permeate BBB and acidic/basic character. pk_a values were calculated solely from the absorption peak at 360 nm as a bathochromic shift was observed from 314 nm at pH 4.5 to 329 nm at pH 9.1. The correlation between *in silico* and *in vitro* pk_a values is found to be considered good. As a result, oximes **25** and **26** are projected to have the maximum amount of non-ionized species (20-30%) and penetrate the BBB at a faster pace. However, protonation of the tertiary amine of these oximes predominantly occurs at physiological pH. All of them were found to have optimal lipophilicity (<5), optimal molecular weight (<500), the optimal number of H-bond acceptors (<10), and H-bond donors (<5) as compared to non-CNS active drugs, which had a lower molecular weight (<450), fewer H-bond donors (<3) and acceptors (<7) moderate hydrophobicity (log P<5), few rotatable bonds, and lower PSA (70A2)[54] (SI Table-8). JR595 was classified as basic at pH 7.4 and 10.5, based on distribution coefficient values. It was discovered that it was metabolically stable. Its absorption into the blood was quick (t_{max} =15 minutes), followed by a quick elimination phase ($t_{1/2}$ = 14 minutes). It was also reported to be a highly permeable compound.[67]

Cytotoxicity

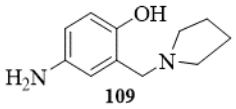
The term cytotoxicity refers to the extent to which a substance can harm a cell. Some substances were tested for cytotoxicity *in vitro*. The cytotoxic effect on cell lines was clearly visible and was deemed to be quite low. BW284c51 (standard compound) was the least toxic compound on all lines. On tested cell lines, the compounds, K298 and K524, do not differ much from the examined standard and can be deemed less toxic. When K298 and K524 were compared to two standards for nicotinic receptor modulation, BW284c51 and edrophonium, they demonstrated a high reversible inhibitory effect (IC50). K524 has the lowest IC_{50} value of 570 ± 5 nm and K298 demonstrated a 4-fold reduced affinity with an IC_{50} = 1900 ± 200 nm. These types of inhibition, when paired with AChE protection, may offer nicotinic receptor protection, especially when used as a pre-treatment. This can also be utilized as a post-exposure treatment due to the inhibition of overstimulated nicotinic receptors. Acute toxicity was assessed for selected compounds in mice (K298, K344, and K474) and rats (K298, K524) using the lethal dose assessment method. It was discovered to be more hazardous than the commonly used AChE reactivator. JR595 seemed to have a moderate cytotoxic effect, whereas HI-6 and 2-PAM had zero cytotoxic effects up to the tested concentration.[67] The dichlorinated analog of K027 showed no cytotoxic effect.

The down selection of these non-oximes was also based on *in silico* evaluation for favorable BBB penetration properties, octanol-water partition (ClogP), and toxicity (rat oral LD50) using the methods implemented in Discovery Studio, DS 2.1, and TOPKAT.[50] As a result, the 10 non-oximes (**59-68**) listed in SI Table 6 were evaluated for BBB penetration, lipophilicity (ClogP), polar surface area (PSA), and molecular weight to study the overall drug-like character of compounds. It has been suggested that molecules with a basic character, molecular weight ~400, and ClogP equals to 4 should have higher permeability, solubility, bioavailability, and CNS penetration. All 10 identified non-oximes appear to have these properties and, as a result, may have therapeutic potential. In addition, unlike oximes, these non-oximes have no charges. SI Table 6 reveals that the ClogP[50] of these non-oximes ranges from -0.035 to 3.66, showing varied BBB penetration abilities. Typically, as ClogP rises, its CNS penetration rises as well, resulting in decreased PSA values. However, because the molecular weight has a stronger influence[68], compounds with a lower molecular weight having correspondingly lower PSA or higher ClogP are preferred for enhanced CNS penetration and permeability. Compound **60** had superior *in vitro* potential as compared to 2-PAM (SI Table 6), while the other nine compounds were found to be 10-fold of 2-PAM.

Compounds **59-62** (SI Table 2), the most effective of the 10 non-oximes, were suggested for *in vivo* assays on Guinea pigs with DFP poisoning. SI Table 7 shows the brain AChE activities, seizure activity, and survival duration of animals after DFP poisoning and treatment. According to the findings, compound **59** displayed very minor changes in the electrical activity of the brain and 24-hour survival with these minor seizures. Compound **60** exhibited persistent seizures during the 24-hour test period. In contrast, compounds **61** and **62** exhibited severe seizures that did not improve with treatment, and all animals died within 7 hours of DFP exposure due to CNS dysfunction. As a result, compounds **61** and **62** did not provide a beneficial effect in terms of reducing the symptoms of OP agent exposure. Leading compound **59** outperformed 2-PAM (SI Table 7) in terms of therapeutic efficacy against DFP-inhibited AChE.

Similarly, Katz et al. discovered a variety of compounds thereby carrying forward the discovery of novel compounds with reactivation potency.[69] Katz's compounds disclosed a high structural resemblance with basic moieties such as pyridine, imidazole, piperazine, or Mannich phenol.[68] As a result, in recent decades, Mannich phenols and general bases have emerged as a new alternative to oxime reactivators. The virtual screening revealed that ADQ, an antimalarial agent, was more active than 2-PAM in reactivating DFP and paraoxon inhibited AChE. However, due to its toxicity, it was ignored. Further research on ADQ revealed that the part responsible for reactivation is ADOC. Katz demonstrated that ADOC, an uncharged, low-molecular-weight molecule, penetrated the BBB better than 2-PAM. The mechanism by which ADOC reactivates DFP-inhibited AChE is unknown. However, both acid-base and nucleophilic mechanisms have been proposed.[70] SI Table 10 shows the proposed mechanism as well as the overall reactivation rate. Cadieux et al.[71] reported a variety of ADOC derivatives by varying the nature, size, and position of substituents soon afterward. On several ADOC analogs, SAR research was undertaken where each benzyl ring substituent was found to have a different magnitude of reactivation, and the reactivation efficacy of each benzyl ring substituent against an OP-inhibited enzyme was estimated (SI Table 11). When the amine group from aniline (**87**) or from the benzylamine (**88**) was removed, the reactivation potency and inhibitory effects on native RHuAChE were both reduced compared to ADOC. These findings may suggest that these substituents are involved in binding rather than reactivation. Likewise, removing the phenol substituent (**89**) made the reactivation functionality ineffective. Furthermore, phenol was blocked as an ether (**90**) which resulted in the reactivation functionality loss against all agents except one (GB), for which the function is considerably reduced in comparison to ADOC. These findings revealed that the phenol substituent is required for reactivation. The reactivation and inhibitory potential against naive RHuAChE were reduced when the benzylic diethylamine on the ring (**91**) was moved to a different place. The inhibitory ability of the molecule against naive RHuAChE was considerably increased by reducing benzylamine (**92**), but this could be neutralized by changing the smaller benzylamine position (**93**) equivalent to that of **91**. As the bulk of benzylamine (**94** and **95**) increases, the inhibitory and reactivation potency decreases. The results revealed that this portion of the molecule is responsible for ADOC binding strength and selectivity. Similarly, the effect of the aniline amine substituent was investigated by raising (**96**) and reducing (**97**) the electron-withdrawing potential, as shown by Hammett values for these substituents. They had higher reactivation efficacy than ADOC, whereas compounds (**98** and **99**) with more electron donating groups were sterically hindered around the phenol and had lower reactivation potency.[72] In conclusion, the phenol substituent was found to be necessary for reactivation. The ring-closed derivatives demonstrated that the ring is responsible for ADOC binding strength and selectivity rather than molecule reactivation capability. In the *in vitro* reactivation assays, however, no compound developed in the study performed better than ADOC. Nevertheless, this paved the way for the discovery of new ADOC derivatives.

MC de koning et al.[73] and 2020[70] synthesized multiple novel Mannich phenols, in the same direction as the identification of ADOC derivatives with reactivation potential toward OP-inhibited AChE. SI Table 12 shows the 50% inhibitory concentration (IC50) for each of the synthesized analogs at various concentrations (1–1000 µM) using human erythrocyte AChE. The majority of the synthesized compounds were simple to synthesize and isolate, but when their reactivation potency against various nerve agents was measured at multiple reactivator concentrations, the results revealed that most of them performed even worse than the previously identified lead compound, ADOC. Even at low concentrations, only the derivative **109** outperformed ADOC, but it was equally ineffective against GA-inhibited AChE. Overall, this pyrrolidine-containing ADOC analogue **109** is the most powerful non-oxime ever discovered till



109

now. In recent times, kinetic parameters have gained popularity as a method for identifying key aspects that influence reactivation potency.

Conclusion and Future Perspectives

Over the past several decades, human beings have been facing the emergence of new alarming disorders.[74] Ever since the discovery of OP nerve agents (1930s), they have been used worldwide. However, over the time, their ill use as a military weapon has alarmed the world. Various cases involving the use of these nerve agents against civilians have been reported in the Syrian Arab Republic Malaysia, UK, Northern Ireland, and several other countries like Syria, Iraq, and Japan. OP poisoning can cause seizures, paralysis, breathing problem, and eventually death. Hence, emphasis must be paid to the discovery of antidotes for OP poisoning with fewer side effects. Recently, the Reactive Skin Decontamination Lotion (RSDL)[75] kit was developed at Defense Research and Development Canada for skin contamination against nerve agents and was approved by FDA. However, it is not yet completely approved for pesticide decontamination. Similarly, various efforts have been made in the discovery of antidotes so far as we have summarized in this paper. Earlier oxime reactivators came into light with their counter effectiveness against the OP agents (pralidoxime, obidoxime, 2-PAM, and HI-6). But as the research continued, they showed some shortcomings. Further novel oximes with better BBB penetration[76], CNS reactivation and uncharged ones[77] gained interest (MINA, DAM, K206, K203, K869, etc.) and were synthesized, and checked for therapeutic efficacy. Even various combinations of already synthesized antidotes were examined which showed elevated results.[78–84] Some natural antidotes[85,86] too came into light having therapeutic abilities. Alternate compounds like Mannich phenols, ADOC and its derivatives, and various other bispyridinium non-oximes were also designed to check their antidotal properties. These compounds developed till date are not all completely effective against all nerve agents as either some show better therapeutic ability against one nerve agent but fail for the other or have some other disadvantages. Most of antidotes have not been tested for BBB penetration. Hence, none of them is approved by FDA. There can be other approaches to improve the BBB penetration which may use certain inhibitors as drug carriers. Current treatment for OP intoxication involves atropine, 2-PAM and diazepam, but none can cure all symptoms fully. At the present time, catalytic scavengers[87,88] and neurosteroids[89–92] are fields of interest in the ongoing work. Anticonvulsant antidotes[93,94] are emerging as a new light to the ongoing tedious work.[95-97] In this review, we presented a detailed discussion on various oxime and non-oxime AChE reactivators showing reactivation and therapeutic ability against organophosphorus nerve agents. Though they are not fully capable of suppressing all symptoms but have been able to reduce lethal effects caused by CWAs. However, there is still hope in the future as various works are going on to synthesize novel antidotes, which may lead to more accurate results. Future advancements in this field will result in better safety from OPs used in both pest control and warfare.

Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Supplementary Information

All tables (SI Table 1 – SI Table 12) are provided in a separate file as supplementary information.

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