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Pralidoxime: a review of its synthesis and antidotal properties against warfare nerve agents

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ABSTRACT: *Acetylcholinesterase (AChE) is an enzyme in the central and peripheral nervous systems that has been studied in fields of research such as that for Alzheimer's and Parkinson's diseases. AChE inhibitors may be either natural or synthetic, which is the case of the organophosphorus compounds, developed as chemical weapons or pesticides, the latter of which is less toxic. The inhibition of organophosphorus is irreversible and is carried out by binding the phosphorus atom to the hydroxyl group of the serine residue within the active site of the AChE, thus preventing AChE from fulfilling its physiological task in cholinergic transmissions, possibly leading to respiratory failure and death. Due to their strong nucleophilic character, AChE reactivators can cleave the bond between the serine residue and the adduct, reestablishing enzymatic activity. This study describes the biological properties and the diverse synthetic methods for pralidoxime, the first AChE reactivator clinically applied.*

KEYWORDS: *Acetylcholinesterase. Antidote. Oxime. Organophosphorus. Pralidoxime. Quaternary reactivator.*

RESUMO: *A acetilcolinesterase (AChE), enzima presente nos sistemas nervosos central (SNC) e periférico, é estudada em pesquisas relacionadas à doença de Alzheimer e à doença de Parkinson. Os inibidores de AChE podem ser naturais ou sintéticos, como os organofosforados desenvolvidos para o uso como armas químicas ou pesticidas, sendo estes menos tóxicos. A inibição por organofosforados ocorre irreversivelmente através da formação de uma ligação entre o átomo de fósforo e a hidroxila do resíduo de serina presente no sítio ativo da AChE. Com isso, a AChE perde sua capacidade de cumprir sua função fisiológica nas transmissões colinérgicas, podendo levar à parada respiratória e morte. Os reativadores de AChE, devido ao seu forte caráter nucleofílico, conseguem romper a ligação entre o resíduo de serina e o aduto, restabelecendo a atividade enzimática. Este trabalho aborda diferentes metodologias sintéticas e propriedades biológicas da pralidoxima, o primeiro reativador de AChE empregado clinicamente.*

PALAVRAS-CHAVE: *Acetilcolinesterase. Antídoto. Oxima. Organofosforado. Pralidoxima. Reativador Quaternário.*

1. Introduction

1.1 Structure and function of acetylcholinesterase and acetylcholine

Acetylcholinesterase (AChE; Enzyme Commission Number [EC] 3.1.1.7) is an enzyme of the hydrolase class, responsible for the hydrolysis of carboxylic acid esters [1], whose active site is composed of a catalytic triad containing serine, histidine, and glutamate residues, with the serine residue being responsible for the attack on the carboxylic ester [2]. Its action occurs in the central and peripheral nervous systems, as well as in the neuromuscular junctions where, together with muscarinic and nicotinic acetylcholine (ACh) receptors, AChE regulates the transmission of electrical impulses (action potentials) along neuromuscular synapses. The physiological function of AChE is the hydrolysis of the neurotransmitter ACh, which ends the action potentials generated by the stimulation of cholinergic receptors. The enzyme acts when ACh is released by the presynaptic neuron in response to an action potential, preventing the accumulation of the neurotransmitter in the synaptic cleft (Fig. 1) [3]–[5].

ACh is transported along the synapse and, when it binds to its receptors, it leads, among other responses, to an influx of K-ions^{+} in the postsynaptic nervous process or in a muscle cell. This process initiates

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action potentials in the postsynaptic cell, which is quickly stopped by the action of AChE by hydrolyzing ACh into its breakdown products, choline and acetate (Fig. 2), which are used to regenerate ACh in the peripheral nerve [3, 4].

Fig. 1 - Structure of a synapse, in which the release and capture of a neurotransmitter occurs.

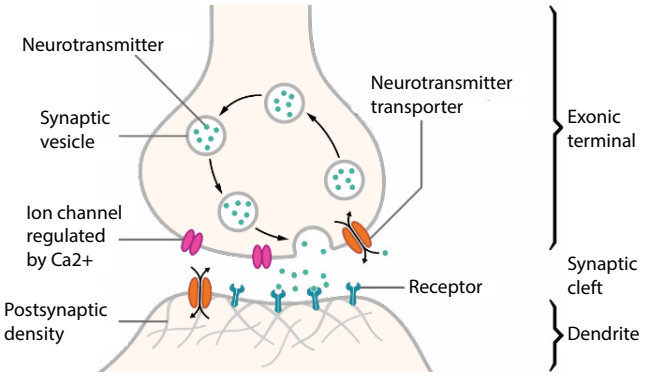

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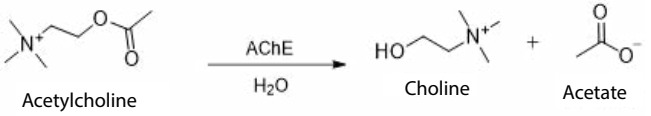
Fig. 2 - Enzymatic hydrolysis of acetylcholine into its precursors: acetate and choline.



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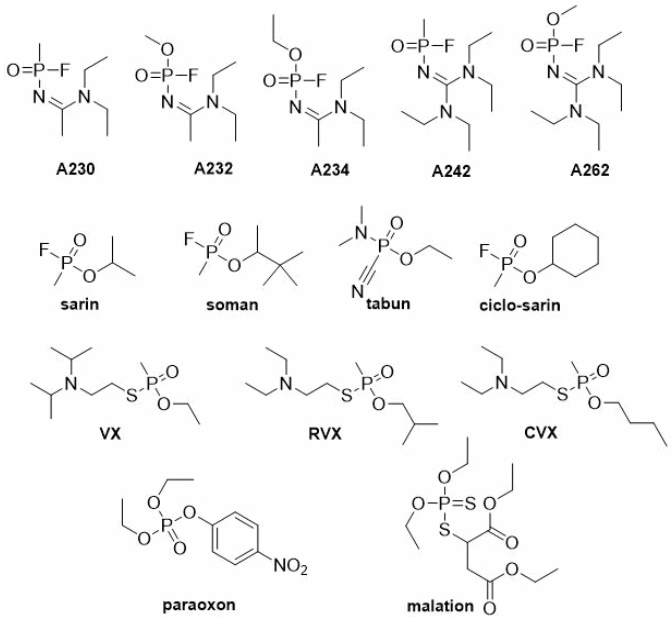
1.2 Inhibition of acetylcholinesterase

Inhibition of AChE causes an accumulation of the neurotransmitter ACh in the postsynaptic cleft, leading to hyperstimulation of cholinergic receptors (muscarinic and nicotinic). This inhibition can be reversible and thus temporary, as is the case with therapies for the treatment of Alzheimer's disease (AD), in which reversible inhibitors such as donepezil, galantamine, and rivastigmine are employed, an approach known as the "cholinergic hypothesis" [7]mainly affecting older people. The unclear root cause and involvement of various enzymes in the pathological conditions confirm the complexity of the disease. Quantitative structure-activity relationship (QSAR. However, in the case of poisoning by pesticides (paraoxon, malathion; Fig. 3) or nerve agents, inhibition occurs irreversibly. Although pesticides are less toxic than neurotoxic agents, both poisonings can lead to malfunction of the central nervous system (CNS) and neuromuscular junctions, which might be lethal [8], [9].

Nerve agents are organophosphorus compounds that can be divided into three series: the oldest, known as the G series (sarin, soman, tabun, and cyclosarin, among others), which are volatile under normal temperature and pressure conditions; the V series (VX, RVX, and CVX, for instance) (Fig. 3), which are more persistent in the environment [10] e.g. in Alzheimer's disease, Parkinson's disease, or in eco-toxicology as a biological marker. Many inhibitors of AChE have been identified in nature as well as prepared in chemical labs as a result of systematic synthetic efforts. The organophosphorus (OP; and the A series, whose substances are known as Novichoks (Fig. 3) [11], which were added to Schedule 1 of the Chemical Weapons Convention (CWC) in June 2020 [12].

Fig. 3 - Structures of G, V, and A series nerve agents and pesticide examples



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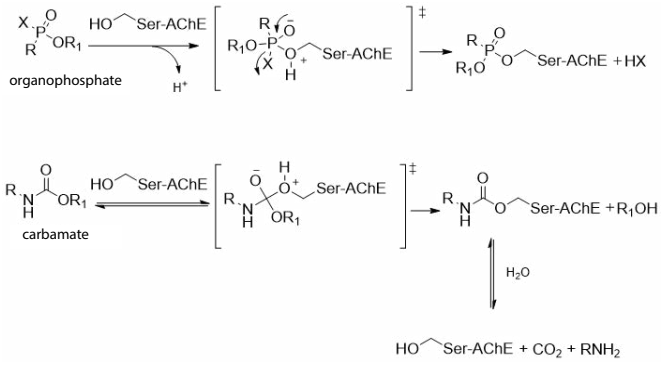
The inhibition of AChE occurs by the formation of a covalent bond between the organophosphate and the hydroxyl of the serine residue at the active site of the enzyme, which makes it impossible for the enzyme to interact with the ACh molecule (Fig. 4). The accumulation of this neurotransmitter results in hyperstimulation of cholinergic receptors, failure of cholinergic synaptic transmission, muscle paralysis, and CNS impairment. These effects constitute a "cholinergic crisis," characterized by symptoms such as miosis (pupil constriction), excessive salivation, bradycardia, diarrhea, emesis, and bronchoconstriction, caused by overstimulation of muscarinic receptors. They also lead to seizures, paralysis, and muscle dysfunction, resulting from the overstimulation of nicotinic receptors. The action on the neuromuscular junctions of the diaphragm's smooth muscle can lead to death by respiratory arrest [13], [14].

The AChE inhibition reaction follows an addition and elimination mechanism (Fig. 4), in which the hydroxyl of the AChE serine residue binds to the electrophilic site of the inhibitor, followed by the elimination of a leaving group. In the case of irreversible inhibitors, such as organophosphates, the leaving group can be, for example, a halogen (fluoride in the case of sarin and soman, Fig. 3) or a cyanide (tabun, Fig. 3). In the case of reversible inhibitors, such as carbamates, the leaving group is an alkoxide [8], [15], [16].

Fig. 4 - AChE reactions with irreversible and reversible inhibitors.



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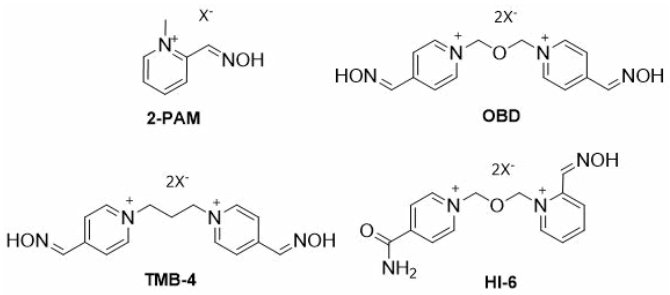
1.3 Reactivation of acetylcholinesterase

To avoid the lethality of organophosphate poisoning, medicines should be administered as soon as possible, especially in cases in which AChE may undergo the "aging" process, in which the antidotes available in the clinic lose their capacity to act, with soman being one of the agents most likely to cause this phenomenon. The rapid drug action also enables the reduction of risks related to neurological issues that impact victims of nerve agents [17], [18]. In addition to an antimuscarinic agent (atropine), which acts by antagonizing the effects of excess neurotransmitter in the synaptic cleft, and an anticonvulsant (diazepam), it is essential to administer an AChE reactivator antidote to reverse the effects of poisoning [19].

AChE reactivators must possess a strong nucleophilic character to break the strong P-O bond between the organophosphate and the serine residue of the AChE catalytic triad. This characteristic is found in cationic oximes derived from pyridine aldehydes, which are the only class of substances used clinically to treat poisoning by nerve agents. Among these, pralidoxime (2-PAM), obidoxime (OBD), trimedoxime (TMB-4), and asoxime (HI-6) are available on the market (Fig. 5) [20]–[23].

Fig. 5 - Clinical acetylcholinesterase reactivators (X^-: Cl^-, I^-, MsO^-).



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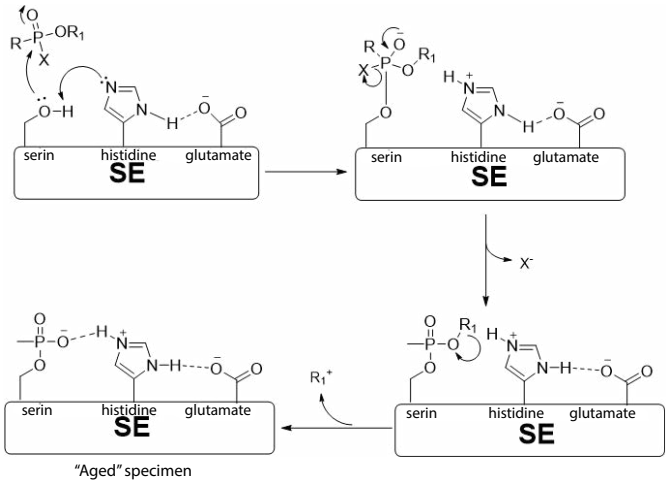
Although these oximes are used in clinical practice, they show limitations, including high toxicity, which restricts their dosage, and a limited spectrum of action against structurally distinct organophosphates, without broad-spectrum reactivation oxime

currently available [13]. Other limitations include their low penetration of the blood-brain barrier due to their cationic nature and their inability to reactivate the "aged" form of AChE, caused by the disproportion of the formed enzyme adduct. The "aging" process consists of the dealkylation of the phosphorus adduct of the inhibited AChE (Fig. 6). To improve the reactivation spectrum of current clinical oximes, one or more of them can be combined when AChE is inhibited by different nerve agents [24], [25].

Fig. 6 - Mechanism of inhibition of AChE by an organophosphate followed by its aging.



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2. Pralidoxime – properties and synthesis

2.1 Biological properties

Synthesized in the United States in 1955 [20], 2-PAM was the first molecule capable of reactivating AChE inhibited by organophosphates to be used in clinical practice. As it is a cationic oxime, it is found in the form of a salt, and can be associated with chloride, iodide, methyl sulfate, or mesylate anions. It is used not only by the Brazilian Army, but also by the armies of the United States, France, and the United Kingdom, in addition to being listed in the Brazilian Ministry of Health's RENAME (Brazilian National List of Essential Medicines) [10], [26], [27]e.g. in Alzheimer's disease, Parkinson's disease, or in eco-toxicology as a biological marker. Many inhibitors of AChE have been identified in nature as well as prepared in chemical labs as a result of systematic synthetic efforts. The organophosphorus (OP.

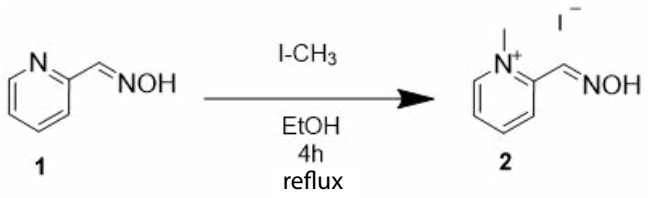
Pralidoxime has been found to demonstrate efficacy in reactivating sarin- or VX-inhibited AChE, especially when combined with atropine [28], [29], but not in reactivating the enzyme inhibited by tabun or soman [30], which reiterates the absence of a "universal antidote" [13]. Another limitation of this reactivator lies in its low rate of penetration in the blood-brain barrier due to the presence of a positively charged nitrogen. Sakurada and collaborators [31] determined that this rate is approximately 10% but later studies suggest that this value is overestimated [32]. This pharmacokinetic limitation is common to all AChE reactivators available in the clinic, which drives the search for new compounds that are increasingly active and efficient for CNS reactivation.

Administration of 2-PAM in humans at a dose of 10 mg/kg resulted in concentrations greater than 4 µg/ml in blood plasma in less than 10 minutes, which was maintained for the subsequent 50–55 minutes due to its high stability in water [33], [34]. The use of this reactivator can include side effects such as dizziness, blurred vision, diplopia (double vision), nausea, and headaches [33], [35].

2.2 Synthetic methodologies for pralidoxime

In 1956, Green and collaborators [36] described a synthesis of pralidoxime iodide (2), in which 2-pyridine aldoxime (1) was reacted with methyl iodide in ethanol under reflux for 4 hours (Fig. 7). The research group also presented a possible interaction between oximes and the nerve agent sarin. The yield of the reaction was not reported in the article.

Fig. 7 - Synthesis of pralidoxime iodide proposed by Green.



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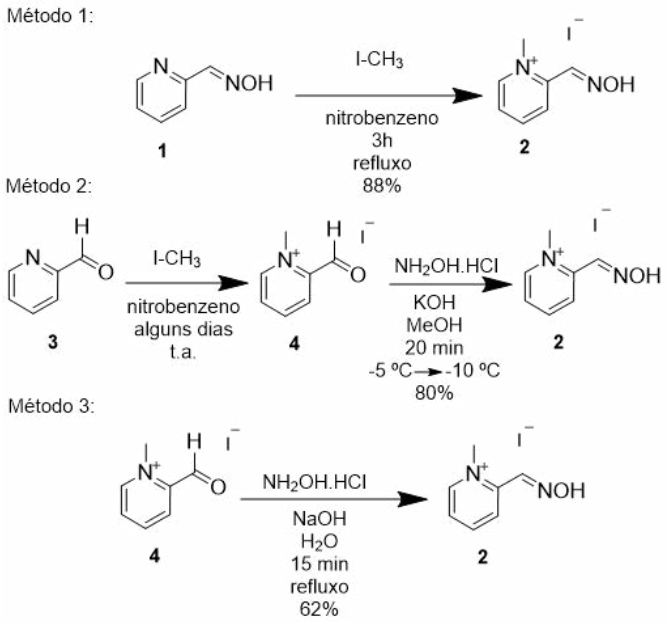
In 1957, Ginsburg and collaborators [37] described the synthesis of pralidoxime and several other derivatives of it. For the synthesis of pralidoxime iodide, three different synthetic routes were presented (Fig.8).

Fig. 8 - The three different synthetic routes for pralidoxime iodide presented by Ginsburg.



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In the first method, **1** reacts with excess methyl iodide in nitrobenzene for 3h with reflux, forming **2** with a yield of 88% (Fig.8) [37].

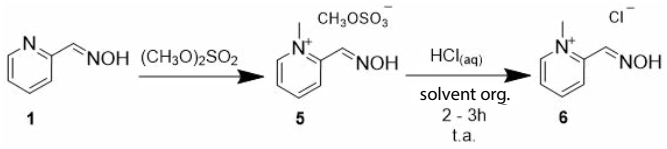
Then, in the second method, 2-pyridinecarboxaldehyde (3) was reacted with excess methyl iodide in nitrobenzene, stirred for a few days at room temperature The product obtained (2-formyl-1-methyl pyridinium iodide, **4**) was precipitated using acetone. In the next step, **4** was added to a methanolic solution of hydroxylamine hydrochloride and potassium hydroxide at −5°C, then stirred for 20 minutes as the temperature dropped to −10°C. The product was precipitated in diethyl ether, obtaining 80% yield (Fig. 8).

In the third method, **4** was reacted with excess hydroxylamine hydrochloride in water under reflux for 15 minutes, followed by pH adjustment to a range of 6–7 using a sodium hydroxide solution. The oxime was recrystallized by methanol or ethanol, reaching a yield of 62% (Fig.8).

In 1964, an innovative route was proposed by Bloch for the synthesis of pralidoxime chloride (**6**) [38]. Previously, **6** was synthesized from the reaction of a solution of **2** with solid silver chloride. Then the silver iodide formed was filtered and the aqueous solution evaporated to dryness at low temperatures. The disadvantage of this method was the residual traces of silver in the product, which were difficult to remove, as well as the use of an expensive reagent, silver chloride. Another method for converting pralidoxime iodide to chloride involved using an anion exchange resin; however, this method was expensive and impractical, as regenerating the iodide-saturated resin was difficult and, similar to the previous method, it required the evaporation of large volumes of water at low temperatures. Direct quaternization of **1** with methyl chloride in a pressure reactor was also performed but the yield was low and successive recrystallizations were required to achieve acceptable purity [38]. Aware of these disadvantages, Bloch proposed a synthetic methodology that involved an intermediate step, forming pralidoxime methyl sulfate (**5**) by reacting **1** with dimethyl sulfate. Molecule **5** was then converted to the chloride by reacting it with concentrated hydrochloric acid and a water-miscible organic solvent (Fig. 9). The solvents tested were isopropanol (85% yield), methanol (30%), absolute ethanol (70%), isobutanol (84%), propylene glycol (27%), dioxane (29%), and acetone (75%). Since **5** proved to be significantly more soluble than

6 in the solvents mentioned, the product could be easily separated by filtration at the end of the reaction and then washed with acetone, achieving a high degree of purity [38].

Fig. 9 - Synthesis of pralidoxime chloride proposed by Bloch.



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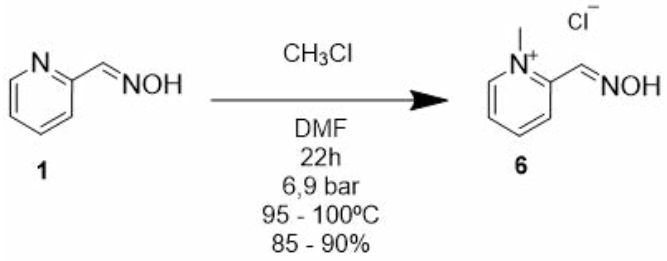
In the work of Ellin and collaborators [39], molecule **6** was synthesized by reacting **1** with methyl chloride in *N*,*N*-dimethylformamide (DMF) (Fig. 10). At atmospheric pressure, both DMF and other solvents such as acetone, ethanol, tetrahydrofuran, and benzene showed low yields for the method presented. However, using DMF at pressures around 7 bar resulted in a yield approximately four times greater than those obtained with the other solvents. This increase is due to DMF being a polar aprotic solvent, and the reaction follows an SN_2 mechanism, which presents, as slow step, a dipolar transition state formation, whose energy is reduced by the solvation effect of DMF, leading to greater stability [39].

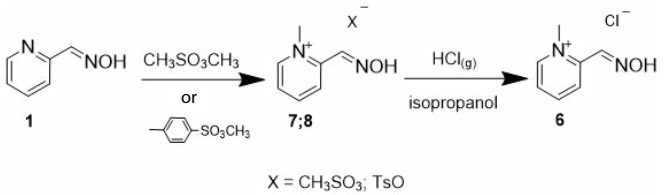
Fig. 10 - Synthesis of pralidoxime chloride proposed by Ellin.



Source: prepared by the authors.

In the study by Rao and colleagues [40], aimed at synthesizing pralidoxime chloride via the methylation of **1**, various methylating agents were tested, including methyl methanesulfonate and p-toluenesulfonate (Fig. 11). For methylation with methyl methanesulfonate, the tested solvents were toluene (70% yield), tert-butyl methyl ether (52%), dimethoxyethane (60%), acetonitrile (90%), and 1,4-dioxane (55%). For methylation with p-toluenesulfonate, the solvents tested were toluene (91%) and acetonitrile (70%). In all reactions, with the exception of methyl methanesulfonate methylation in acetonitrile, the crude product crystallized in an ethanol solution with ethyl acetate. In the final step, to convert to pralidoxime chloride, a solution of the methylation product in isopropanol was bubbled with anhydrous hydrogen chloride gas to form **6** (Fig. 11).

Fig. 11 - Synthesis of pralidoxime chloride proposed by Rao.



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3. Discussion

The 2-PAM is part of a broad family of compounds with strong nucleophilic characteristics, capable of reactivating AChE inhibited by neurotoxic agents. From a structural standpoint, it is used as a quaternary ammonium salt and shows only one oxime group, whereas the other three available reactivators show two. The quaternization of oximes aims to increase their affinity for the anionic catalytic site of AChE, enhance water solubility, and adjust the pKa values (from 7.0 to 8.35) to facilitate the reactivation process [41]. A major limitation of cationic oximes is their poor penetration of the blood-brain barrier due to their low lipophilicity, which causes them to act predominantly in the peripheral nervous system [42]. However, 2-PAM showed a 10% penetration of the blood-brain barrier in rats, compared to 1 to 3% for the bipyridinium oximes [31],

[43]. Among the available antidotes, asoxime is the least toxic, followed by 2-PAM, whereas obidoxime and trimedoxime are the most toxic [44].

Although clinical antidotes are cationic and have one or two pyridinium rings, recent studies have explored AChE reactivators with different structural characteristics. In search of greater lipophilicity, neutral oximes and oxime derivatives containing nitrogen heterocycles in their structure have been evaluated as potential new classes of AChE reactivators [45]–[47]. Other approaches with the same objective include structural modification of cationic oximes by adding fluorine atoms to the pyridinium rings and transforming these oximes into prodrugs—drugs administered in an inactive form and activated by biotransformation within metabolic pathways of the body [48]. In addition to chemical approaches, research have been exploring new methods of administering antidotes, developing techniques that facilitate the entry of reactivators into the central nervous system, such as intranasal administration [49].

Regarding the synthesis methodologies presented, considering the number of steps and yield, the most efficient synthetic methods were those reported by Ginsburg (one step; 88% yield) and Ellin (one step; 85–90% yield).

4. Conclusion

Several synthetic methodologies for 2-PAM have been described, making it one of the most relevant pyridinium oximes available for combating poisoning by nerve agents. We searched for articles published since 1955, the year 2-PAM was first reported. In the discussion, the synthetic routes were compared in terms of efficiency, considering the number of steps, yield, and feasibility of the syntheses. A comparison was also made between the properties and limitations of 2-PAM relative to other AChE reactivators. Approaches were also cited in the search for new classes of AChE reactivators.

List of abbreviations and acronyms

2-PAM	=	Pralidoxime
ACh	=	Acetylcholine
AChE	=	Acetylcholinesterase
CWC	=	Chemical Weapons Convention
AD	=	Alzheimer's disease
DMF	=	*N*,*N*-dimethylformamide
EC	=	Enzyme Commission Number
HI-6	=	Asoxime
OBD	=	Obidoxime
RENAME	=	Brazilian National List of Essential Medicines
SNC	=	Central Nervous System
TMB-4	=	Trimedoxime

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