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Preparation of New Acetylcholinesterase Reactivators with an Amidoxime Functional Group and their In Vitro Testing

(diploma thesis)

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I here declare that this diploma thesis is an original author's copy, worked out by myself. All literature and other sources used in this thesis are quoted and listed in the literature section.

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1. ABBREVIATIONS

- ACh acetylcholine
- AChE acetylcholinesterase
- ATChCl acetylthiocholine
- BChE butyrylcholinesterase
- ChE plasma cholinesterase
- CVX chinese VX
- EtOA ethyl acetate
- HI-6 asoxime
- LüH-6 obidoxime
- MeCN acetonitrile
- OPC organophosphorus compounds
- PAM-2 pralidoxime
- SD standard deviation
- TMB-4 trimedoxime
- VR russian VX

2. AIM OF THE WORK

Organophosphorus compounds are very dangerous substances, which were many times in the past used in wars or in brutal terrorist attacks. Their simple structure, relatively easy and not so expeansive synthesis makes them even more dangerous. Theire toxicity lays in the irreversible inhibition of AChE leading to hyperreactivation of choline system with all the negative symptoms, which leads to death unless reactivator is applied. Even though it is some time when the first substance was invented there are not many potent reactivators of AChE.

Oximes proved to be efficient in breaking chemical bonds between organophosphorus and AChE. Thus many analogs are being synthesized in hope of finding more efficient substances.

Mono and bisqvartenary substances are quiete frequently used as a basic structure for new oximes. Also in this work mono and bisqvartenary structures were used for basic scelet. Oxime group was modified on carbon atom with $-NH_2$ group, and nitrogen atom in pyridine underwent modification by numerous methylene groups. Every synthesized substance differed in one homologous unit of $-CH_2-$.

Expectations were for either better reactivation or inhibition than that of already widely used substances. Unfortunately as can be seen in the experimental section neither of these expectations proved to be valid.

Although none of the substances have the potential to be used in clinical practice it is important to show which structure modifications do not have positive effect on activity, so no more money is wasted on their farther research.

3. INTRODUCTION

3.1. CHOLINESTERASES

In 1914 Henry Hallet Dale differentiated between the muscarine and nicotine actions of choline esters on different tissues. He proposed that esterase is the main factor that contributes to the removal of acetylcholine from the circulation. His hypothesis was based on observations of the inactivation of acetylcholine (ACh) injected into cats. However, it wasn't until 1926 that Loewi and Navratil, working on isolated frog's hearts, demonstrated its existence by inhibition with physostigmine, thus prolonging the effect of ACh. In 1932 Stedman prepared an extract from horse serum, capable of splitting ACh, which was called cholinesterase.

Nowadays, enzymes which preferentially hydrolyse acetyl esters such as ACh are called acetylcholinesterase (AChE) or acetylcholine acetylhydrolase (EC 3.1.1.7), and those which prefer other types of esters such as butyrylcholine are termed butyrylcholinesterase (BChE) or acylcholine acylhydrolase (EC 3.1.1.8). BChE is also known as pseudocholinesterase, non- specific cholinesterase, or simply cholinesterase. (1) In human body we can find different types of cholinesterases, which differ in their location in tissues, substrate affinity and physiological function. The two main types are plasma cholinesterase (ChE) present in plasma, liver, cerebrospinal fluid and glial cells, and AChE located in the nervous system and also in the outer membrane of red blood cells. (2, 3)

3.1.1. **Plasma cholinesterase (butyrylcholinesterase)**

Plasma cholinesterase, known also as BChE, is a circulating plasma glycoprotein synthesized in the liver. This enzyme does not perform any known physiological functions. Recently it was indicated that BChE may be involved in cholinergic transmission, neurodegenerative disorders and other nervous system functions such as cellular proliferation and neurite growth during the development of the nervous system. (2, 3)

BChE is a serine hydrolase that belongs to the esterase family and catalyses the hydrolysis of esters of choline including acetylcholine. Compared to AChE, function of BChE is not very known, in spite of today's scientific progress. BChE is able to split down similar substances as AChE does and it can act as AChE in the case of Alzheimer's disease. Due to these mentioned facts, hypothesis exists that AChE reactivators used for treatment of nerve agent and pesticide intoxications could reactivate inhibited BChE as well, and so BChE can act as a scavenger agent to sequester the molecules of nerve agents or pesticides from the blood stream. (4)

3.1.2. **Acetylcholinesterase**

AChE occurring in mammals but also in other species, bacteria and some plants is an enzyme playing an essential role in cholinergic nerve transmission. It splits neuromediator acetylcholine released at peripheral and central cholinergic synapses. AChE activity and inhibition varies in different tissues and organs. It exists in excess in different structures including brain. The brain is very heterogeneous but complex

organized structure of the body. Generally, the brain AChE activity is many times higher than it is necessary for its physiological function. (5)

The main function of AChE is the rapid hydrolysis of the neurotransmitter ACh at cholinergic synapses. The hydrolysis reaction proceeds by nucleophilic attack of the carbonyl carbon, acylating the enzyme and liberating choline. This is followed by a rapid hydrolysis of the acylated enzyme yielding acetic acid and the restoration of the esteratic site. (1)

The active site of AChE is considered to contain two subsides: a negatively charged (anionic site) to which the positively charged quaternary nitrogen moiety binds and an esteratic site containing the actual catalytic residues, probably both an electrophilic and a nucleophilic group. A second 'anionic' site, which became known as the 'peripheral anionic' site, around 14Å from the active site, was proposed on the basis of binding of bisquaternary compounds. (1)

O'Brien (1969) submitted his 'heretical thought' that no true anionic site existed. He argued that the carbon analogue of ACh which lacks the charge of the quaternary nitrogen atom, and other uncharged molecules, are good substrates for the enzyme. He also noted that if columbic forces were of the major importance in substrate binding, then the ammonium ion should be a very good inhibitor, whereas it is one of the poorest. (1)

The nucleophile was assumed to be a serine residue, with a histidine residue enhancing its nucleophilicity. As other enzyme mechanisms became understood, AChE was classified as a serine hydrolase, and therefore assumed to contain a catalytic triad of Asp-His-Ser at the esteratic subsite. A great step forward in the understanding of the catalytic mechanism, and mode of action of inhibitors, came in 1991 with the determination of the three dimensional structure of dimeric *T. californica* AChE. (6) The structure determination uncovered several interesting findings. The catalytic triad seemed to consist of a glutamate residue, rather than the usual aspartate. The active site

was discovered to be located 20Å from the enzyme surface at the bottom of a narrow gorge, lined with 14 aromatic residues, which may be important in guiding the substrate to the active site. There was no discernible 'anionic' site, the quaternary nitrogen of choline binds chiefly through interactions with the π electrons of the residue Trp84. The structures of AChE with the bound inhibitiors; decamethonium, tetrahydroaminoacridine, edrophonium, and 1,5-*bis* (4-allyldimethylammoniumphenyl)pentan-3-one-dibromide have been determined. These show that ligands at the peripheral site also do so by interaction with π electrons, in this instance with the residue Trp279. (1)

3.2. ORGANOPHOSPHORUS COMPOUNDS

Organophosphorus compounds (OPC) have been used as pesticides (chlorpyrifos, malathion, and parathion) and as warfare nerve agents (tabun, soman, sarin, DFP, paraoxon and VX). Exposure to even small amounts of an OPC can be fatal. (3) Intoxication by OPC usually results from oral intake, whereas inhalation or dermal absorption does not play a major role.

Most common cause of pesticide poisonings is due to occupational exposure. However, pesticide poisonings associated with high mortality rates are usually the result of self-poisoning. Numerous self poisoning incidents were detected due to the availability of acutely toxic pesticides used in agriculture. Organophosphorus pesticides cause approximately hundreds of thousands deaths per year worldwide. Due to the high toxicity, OPC bear a potential risk for use in wars and terrorist attacks. Namely, use of nerve agents during Iran-Iraq war in the 1980's and during terrorist attacks by the Aum Shinrikyo sect in Japan emphasizes the constant threat and need for awareness of the future homicidal attempts, as well as the urgent need for the development of an effective medical treatment. (8)

Death is usually caused by respiratory failure resulting from paralysis of diaphragm and intercostals muscles, depression of the brain respiratory center, bronchospasm and excessive bronchial secretions. (3)

3.2.1. **History**

In the middle of the 19th century first synthesis of an OPC was performed by Moschnine in a laboratory in Paris. Tetraethylpyrophosphate was the first OPC ever synthesized and was published in 1854 by de Clermont. In 1873, von Hofmann synthesized an OPC with direct phosphorus-carbon linkage (methylphophoryl dichloride). Later Lange and von Kreuger presented dialkylphosphonofluoridates obtaining many OPC's with fungicidal or insecticidal features. (8)

Between 1934 and 1944 Schrader's team introduced approximately 2000 OPCs, two well known organophosphorus insecticides diethyl-*p*-nitrophenylphosphate (paraoxon) and diethyl-*p*-nitrophenylthiophosphate (parathion). In that time Nazi Germany insisted on redefining Schrader's aim from organophosphorus insecticides to organophosphorus chemical warfare agents such as tabun, sarin and soman. Besides fluorine containing esters (sarin and soman) Schrader also developed, fluorine containing, diisopropyl phophorofluoridate (DFP). During this same period British and American scientists were assessing the toxic properties of DFP and other alkyl phosphorofluoridates. DFP as a potential chemical warfare agent became a model substance used for studying cholinergic transmission. The fourth chemical warfare agent VX was synthesized later, in 1957, by Tammelin. (8)

During the same time Russian scientists performed the so-called Russian VX (VR). Another structural analog of VX known as Chinese VX (CVX) was also developed. Vitro experiments using rat, pig and human brain compared VX agent with VR and CVX. Examination of these substances towards human and pig AChE demonstrated the following inhibitory potency: VR>CVX>VX. In the late 1980s and 1990 Russian chemists produced some new agents including Novichok-5 and Novichok-7. According to these scientist Novichoks are nerve agents, which become lethal only after mixing otherwise

benign components and their toxicity is 10 to 100 times higher than one of the other nerve agents. (8)

After World War II OPCs were introduced for pesticide use, as originally intended. Later numerous other OPCs have been synthesized. Currently more than 100 structurally different OPCs are used mainly as insecticides in gardening and agriculture. (8)

3.2.2. **Warfare nerve agents**

3.2.2.1. Tabun

Tabun (ethyl dimethylphosphoramidocyanidate) *(I)* causes centrally mediated seizures that can progress to status epilepticus and contribute to profound brain damage. Therefore the ability of antidotes to counteract the acute neurotoxic effects of tabun and prevent irreversible lesions in the central nervous system is very important for efficient antidotal treatment of acute tabun poisonings. (9)

The effect of tabun can be probably antagonized due to the changes in hydrogen bonding and the conformational changes of AChE-tabun complex prior to an aging process in the active site of AChE. (10)

The unsatisfactory efficacy of oximes to eliminate tabun induced neurotoxicity can be explained by very low potency of oximes in reactivating tabun inhibited AChE. The reason for this low reactivation potency seems to be the presence of a free electron pair located on amidic nitrogen, which makes the nucleophilic attack of oximes impossible. TMB-4 was the first efficient oxime in the treatment of tabun intoxication. Obidoxime is probably the most suitable oxime for the antidotal treatment of acute tabun exposure. On the other hand, obidoxime is not efficient for the treatment of soman poisonings because it does not reactivate successfully soman inhibited AChE, it can even counteract the beneficial effects of atropine. (9)

According to new studies, AChE reactivators with an oxime group in the position four at the pyridinium ring are the most potent reactivators of tabun inhibited AChE. Another important property is the number of methylene groups linking the chain between two quaternary pyridinium rings in the molecule of reactivators. Therefore monopyridinium oxime pralidoxime is not able to reactivate tabun inhibited AChE at all, bispyridinium oximes, such obidoxime and trimedoxime, seem to be efficacious reactivators of tabun inhibited AChE. (11)

Another experiment showed that three new bispyridinium quaternized oximes K027, K048 and K074, and TMB-4 were promising in reactivation of tabun phosphorylated AChE not only in combination with atropine but also as pretreatment drug. Their very good antidotal efficacy makes them interesting for future investigation. (12)

Recent studies revealed that the newly developed oxime K-203 is more effective in reactivation of tabun inhibited AChE than currently available obidoxime and trimedoxime. K-203 is suitable for the replacement of these oximes in the treatment of tabun intoxications. (13)

3.2.2.2. Soman

The respiratory failure resulting from soman(3,3-dimethylbutan-2-yl methylphosphono-fluoridate) *(II)* poisoning is very difficult to treat effectively because soman causes respiratory failure by inhibiting AChE in the central nervous system, and oximes do not penetrate the blood brain barrier in sufficient concentrations. Soman appears to cause centrally mediated seizures that can progress to status epilepticus and contribute to profound brain damage. Also dealkylation, the mechanism of ageing, occurs rapidly after inhibition of AChE by soman. This aging causes considerable difficulties in the reactivation of soman inhibited AchE. (14)

According to numerous experiments, bispyridinium aldoximes such as HI-6 and HLö-7 have shown better efficacy against OPC intoxication than the monopyridinium oximes 2-PAM and P2S. HI-6 and HLö-7 have several structural similarities, one aldoxime group in one pyridinium ring and one carboxamide group on the other pyridinium ring. HLö-7 bears an additional aldoxime group in position four on one of the pyridinium ring. This similarity in the chemical structure of HI-6 and HLö-7 probably plays an important role in the acetylcholinesterase reactivation during soman intoxication. (15)

Two newly developed oximes (K-74 and K-75) were suggested for antidotal treatment after exposure to soman. These oximes are not suitable for the poisoning treatment of soman, because of their absence of neuroprotective effects. The neuroprotective effects of antidotes (atropine, obidoxime, obidoxime/atropine mixture) on rats poisoned with soman were studied. Atropine combined with obidoxime and alone seems to be quite

effective antidotal treatment for the elimination of soman-induced neurotoxicity, although the antidotal mixture is significantly less effective than atropine alone because obidoxime can counteract the beneficial effects of atropine. Obidoxime appears to be practically ineffective to decrease soman-induced neurotoxicity. The neuroprotective effects of antidotal mixture with atropine and obidoxime depend on the antimuscarinic effects of atropine only. Thus, the replacement of obidoxime with more effective AChE reactivators is necessary to increase the neuroprotective efficacy of antidotal treatment in case of soman poisonings. Nowadays, the HI-6 oxime alone is still the best AChE reactivator for the antidotal treatment of intoxication with soman. (16, 17)

The unsatisfactory studies have prompted pretreatment possibilities for organisms exposed to nerve agents. Prophylactic effect of pyridostigmine can result from its relatively irreversible inhibition AChE. However, pyridostigmine-induced increase in the level of ACh can itself cause symptoms of poisoning. The addition of centrally acting anticholinergic drugs to pyridostigmine for the pretreatment of acute soman exposures seems to be rational because a mixture of pyridostigmine with anticholinergic drugs would be able to increase the resistance of soman-poisoned animals and eliminate side effects of pyridostigmine, especially the effects of accumulated ACh. The combination of prophylactic antidotal mixture containing pyridostigmine and centrally active anticholinergic drugs with common antidotal treatment consisting of anticholinergic drug (mainly atropine) and oxime (mainly obidoxime, pralidoxime or HI-6) seems to be sufficiently effective to counteract acute neurotoxic effects of soman. (18)

3.2.2.3. Sarin

From the chemical point of view, sarin is propan-2-yl methylphosphonofluoridate *(III)*. Few studies showed that monoquaternary reactivators are unable to reactivate sarin inhibited AChE. Only bisquaternary compounds derived from HI-6 or obidoxime should be designed as potential cholinesterase reactivators. Only obidoxime and HI-6 achieved good reactivation potency at a level safe for humans (0.0001 M). Moreover HI-6 seems to be the best reactivator at this concentration. HI-6 provided same results for cyclosarin (*O*-cyclohexyl methylfluorophosphonate) inhibited AChE. Unfortunately this oxime cannot be considered as a broad spectrum reactivator, because it is not able to reactivate tabun inhibited AChE. (19)

3.2.2.4. DFP

DFP (dipropan-2-yl phosphorofluoridate) *(IV)* was investigated in the 1940´s by McCombie and Sauders in the United Kingdom. This OPC has neuropathic potency, i.e. capability of inducing delayed neuropathy. (20)

Newly developed K-oximes K-107, K-108 and K-113 showed effective *in vitro* protection of DFP inhibited AchE. Unfortunately, these oximes are *in vivo* more toxic than all the other oximes, therefore can be only administered at a low dosage, which is insufficient to protect from DFP induced mortality. Better *in vivo* reactivation characteristics were presented by 2-PAM and K-27. (21)

3.2.2.5. Paraoxon

Paraoxon (diethyl 4-nitrophenyl phosphate) *(V)* has not been used for military purposes. It is the neurotoxic metabolite of the insecticide parathion (*O*,*O*-diethyl *O*-(4-nitrophenyl) phosphorothioate). Methoxime, trimedoxime, pralidoxime and obidoxime were efficient in reactivation of paraoxon inhibited AChE. (20)

For reactivation of paraoxon inhibited AChE, one aldoxime group in position four on heteroatomic ring is needed. The other substituent at second heteroatomic ring influences the reactivation ability by different affinity to AChE. Carbamoyl functional group gave better results for paraoxon inhibited AChE than cyano group. Length and structure of connecting chain is another important factor for activity of the reactivator. (22)

3.2.2.6. Malathion

Malathion (diethyl 2-{[methoxy(methylsulfanyl)phosphoryl]sulfanyl}butanedioate) *(VI)* is an organophosphorus pesticide activated by the cytochrome P450 system by oxidative desulfuration to form malaoxon, an effective inhibitor of AChE. A study demonstrates that bispyridinium oximes K-27 and K-48 are able to reverse malathion induced AChE inhibition. (23)

3.2.2.7. VX

In the case of VX (*S*-[2-(dipropan-2-ylamino)ethyl] *O*-ethyl methylphosphonothioate) *(VII)* inhibition, obidoxime and new oximes K-27 and K-48 seem to be the best AChE reactivators. (24)

3.2.3. **Mechanism of action of OPC**

The basic chemical structure of OPC is described by Schrader's formula. The structure of OPC differs in substituents at the phosphorus atom. Substituents R^1 and R^2 are represented by alkyl, alkoxy, alkylthio or amino groups and substituent X is formed by a labile acyl residue (halide, cyano, phenol or thiol group) known as the leaving group. The reactivity of OPC varies upon the chemical structure. Electrophilicity of the phosphorus atom is principal for the biological actions of OPC. Organophosphates and phosphonates having double bond between phosphorus and oxygen atom are highly electrophilic at the phosphorus atom and are highly reactive. Groups that enhance the reactivity of the phosphorus atom are nitro, cyano, halogen, ketone and carboxylic ester. Deactivating groups include hydroxyl and carboxylic acid. (8)

The mechanism of action of OPC consists in the inhibition of serine esterases, primarily acetylcholinesterase and butyrylcholinesterase. OPC are electrophilic molecules with the potency for phosphylation (denotes phosphorylation and phophonylation) the serine hydroxyl group located at the active site of AChE. This phosphylation occurs by the loss of the leaving group and formation of a covalent bond with AChE through the serine hydroxyl. The resulting phospholated AChE is generally very stable and is slowly regenerated by spontaneous hydrolysis of the ester. While the AChE remains phosphylated, its enzyme activity is repressed, and ACh accumulates in the synaptic clefts of muscles and nerves, thus causing overstimulation of cholinergic receptors. Additionally, OPC may interact with other serine esterases, leading to direct action on muscarinic and nicotinic receptors possibly modulating functions of these receptors and induce organ lesions. Propyl, isopropyl, butyl and higher alkyl phosphates and phosphonates are more likely than methyl or ethyl analogs to act with secondary targets. The concentrations of OPC acting directly on nicotinic receptors are much higher than those on muscarinic receptors, suggesting that muscarinic receptors are more important as secondary targets in OPC action. Combination of possible interactions may produce resultant toxic effects for the particular OPC. The spectrum of effects is further modulated by various toxicokinetic factors. (8)

Phase I of metabolic transformation of OPC consists of six major reactions, most of which are activation pathways:

(1) oxidative desulfuration – conversion of thiophosphate to the more toxic oxon form (parathion, malathion, fenitrothion, diazinon, dimethoate, azinphosmethyl, fonofos)

(2) oxidative *N*-dealkylation (dicrotophos, monocroptophos, dimethoate, phosphamidon)

(3) oxidative *O*-dealkylation (chlorfenvinphos)

(4) thioether oxidation (disulfoton, phorate, fenthion, demethon-*S*-methyl)

(5) side chain oxidation (fenitrothion, diazinon)

(6) various nonoxidative reactions (trichlorfon, naled)

In phase II (the detoxification phase) OPCs undergo conjugation with endogenous substrates such as glucuronides and sulfates. OPC are detoxified by the action of:

(1) carboxylesterases (malathion, fentoate, soman, sarin, tabun)

(2) phosphorylphosphatases (pyrimiphos methyl, diazinon, paraoxon, DFP, soman, sarin, tabun)

(3) glutathione redox system (methyl parathion, methyl paraoxon, fenitrothion, diazinon, dichlorvos, bromophos, chlorfenvinphos, mevinphos)

OPC are activated by the cytochrome P450 system, NADPH-cytochrome P450 reductase and flavin containing monooxygenases.

Carboxylesterases are important in the detoxification of OPC via hydrolysis of ester bonds in OPC on the carboxyl ester side chain, as well as binding OPC at the active site of the carboxylesterase, which reduces the concentration of OPC potentially inhibiting AChE. (8)

The most common pathway for the metabolism of OPC is via hydrolysis that results in removal of the more labile leaving group. Products are excreted in the urine and serve as biomarkers of exposure. These biomarkers include dimethylphosphate, dimethylthiophosphate, dimethyldithiophosphate, dimethylphosphorothioate, diethylphosphate, diethylthiophosphate, diethyldithiophosphate and diethylphosphorothioate. Because insecticidal OPCs differ mostly in the structure of the leaving group, the rates of hydrolysis differ from compound to compound, despite similarity in some of resulting urinary metabolites. The phosphorylphosphatases involved in hydrolysis of OPCs, and therefore inactivation are present in many tissues and have high activities in liver, intestine and plasma. (8)

3.2.4. **Toxic effects of OPC**

Activation or detoxification depends on the chemical structure of the OPC and its interaction with enzyme systems. Toxicity is determined by the relative prevalence and intensity of the metabolic pathways. Exposure to OPC can induce different clinical syndromes:

(1) acute cholinergic crisis as a result of AChE inhibition

(2) intermediate syndrome whose mechanism is still unclear

(3) organophosphate-induced delayed neuropathy caused by the inhibition of neuropathy target esterase

(4) chronic organophosphate induced neuropsychiatric disorder due to long-term low-level exposure

Acute cholinergic crisis immediately follows exposure to OPC and includes signs and symptoms resulting from hyperstimulation of

(1) muscarinic receptors such as bradycardia, bronchoconstriction, bronchorrhoea, abdominal cramps, increased gastrointestinal motility, hypotension, miosis, hypersalivation

(2) nicotinic receptors such as hypertension, fibrillation, tachycardia

(3) both central muscarinic and nicotinic receptors, such as movement incoordination, tremor, seizures, central depression of respiration, coma, death. Death is usually due to asphyxia, followed by hypoxemia and cyanosis. Cause of death can also be cardiac arrest or heart failure, by far

central respiratory failure is the most frequent cause of death after intoxication with OPC.

3.2.5. **Clinical aspects of acute OPC poisoning**

Three types of signs and symptoms of acute poisoning with OPC can be manifested:

(1) muscarinic

(2) nicotinic

(3) central effects

Table 1 **SIGNS AND SYMPTOMS OF POISONING WITH OPC (3)**

Signs and symptoms		Severity of poisoning
Muscarinic	Nausea, vomiting, diarrhea, bradycardia, bronchoconstriction, increased bronchial secretion, salivation, sweating	Mild
Central	Headache, diziness	
Muscarinic	As above plus miosis, urinary/faecal incontinence	Moderate
Nicotinic	Muscle fasciculations, tachycardia	
Central	As above plus ataxia, mental confusion	
Muscarinic	As above	Severe
Nicotinic	As above plus muscle fasciculations in respiratory muscles	
Central	As above plus coma, tremor, convulsions, bronchospasm, seizures, death due to respiratory depression	

The duration of these effects is determined by the properties of the compound: liposolubility, stability of the OPC-AChE complex and reactivation after the use of cholinesterase reactivators such as oximes. It is important to know that only OPC containing P=O bond (direct inhibitors) are potent AChE inhibitors, while those having P=S group (indirect inhibitors) must be metabolically formed to P=O group. The signs and symptoms of poisoning with direct inhibitors appear quickly during or after exposure, while those with indirect inhibitors appear slowly and last longer. (3)

Clinical diagnosis is based upon medical history, exposure circumstances and the presence of poisoning symptoms. Confirmation of diagnosis can be tested by measurement of red blood cell AChE or plasma ChE, which are biomarkers of toxicity of OPC and carbamates. Red blood cell AChE presents the same as the enzyme in the target synapses and its levels are assumed to reflect the effects in target organs. However, it must be concerned that the assumption is only correct when the inhibitor has equal access to blood and synapses. Since access to blood is always easier than the access to brain, the inhibition in red blood cells may be overstimulated relative to that in brain. (3)

3.3. TREATMENT OF OPC POISONING

The current treatment of OPC poisoning includes the use of three types of drugs:

(1) anticholinergic drug (atropine)

(2) cholinesterase-reactivating agents (oximes)

(3) anticonvulsant drugs (benzodiapines)

Atropine was developed as a first antidote for its parasympatolytic effects. Atropine was isolated from *Atropa belladonna* L. by Main in 1835. In 1863 it was used against cholinesterase-inhibiting agent by Fraser. After a remark that OPC could act toxic in a similar manner as carbamates (including physostigmine), the use of atropine was suggested in the treatment of OPC poisoning. Atropine, as an antimuscarinic drug, is not able to inhibit effects provoked by nicotinic overstimulation, while it possesses limited antimuscarinic action in the central nervous system (CNS). Convulsions can be blocked with atropine for limited time after exposure to OPC since other systems (glutamate and γ-aminobutyric acid) have become involved in cholinergic hyperstimulation in the brain. Nevertheless, atropine is still beeing used as the initial drug of choice in OPC poisoning. (8)

In present the most important anticonvulsant is diazepam. The combination of atropine and diazepam is more effective than atropine or oxime alone in reducing mortality. Benzodiazepines potentiate the action of the inhibitory neurotransmitter γ-aminobutyric acid at its receptors. Diazepam probably decreases the synaptic release of ACh. The main consequence of the action of benzodiazepines in CNS is hyperpolarization of neurons which makes them significantly less susceptible to cholinergically-induces depolarization. The ultimate result is cessation of propagation of convulsions. Diazepam affects also anxiety, restlessness and muscles fasciculation, terminates convulsions, and reduces morbidity and mortality when used in conjuction

with atropine and oxime. Whenever convulsions are present, diazepam should be given to patients poisoned with OPC. (8)

3.3.1. Oxime therapy

The first in vitro destruction of phosphylated AChE was achieved with hydroxylamine in 1951. In the following years, development of specific antidotes, originally aimed to reactivate AChE inhibited with nerve agent, has been based on the hypothesis that successful regeneration of inhibited enzymes could be achieved by the nucleophilic attack of a substance structural similar to ACh meaning a presence if quarternary ammonia group in a molecule of reactivators. The first pyridinium oxime pralidoxime (PAM-2) could reactivate the phosphylated enzyme much faster than hydroxylamine. In 1956, PAM-2 was administered against parathion poisoning in Japan. Further investigations came up with synthesis of bispyridinium oximes, such as trimedoxime (TMB-4) in 1957, obidoxime (LüH-6) in 1964, asoxime (HI-6) in 1967 and HLö-7 in 1986. (8)

The antidotal potency of oximes consists in their ability to reactivate the phosphorylated or phosphonylated cholinesterases. Oximes act by displacing the phosphorus moiety from the enzyme due to their nucleofilicity and affinity for the enzyme. In the reactivation process the enzyme activity is restored, the enzyme is liberated in its active form, and phosphylated oximes are formed. Reactivation is possible only if the ester substituents on the phosphorus are not hydrolyzed and if aging of the phosphylated enzyme does not occur. The process depends on the structure OPC bound to the enzyme, the source of the enzyme, the structure and concentration of the oxime presented in the active site and the rate of aging (post-inhibitory dealkylation). Phosphylated oximes built up during the reactivation might be potent inhibitors of cholinesterases that could cause re-inhibition of the reactivated

enzyme. Oximes bind to AChE as reversible inhibitors and form complexes with AChE either on the active, allosteric or on the both sites of the enzyme and protect AChE from phosphylation. When the reversible inhibitor binds to the active site, the competiton is between the OPC and reversible inhibitor. Binding of an inhibitor to the allosteric site induces indirect protection of the active site. (3)

There are five important structural factors that influence the affinity of reactivators toward inhibited AChE and oxime reactivation:

1) the presence of quaternary nitrogen in the reactivator molecule
2) the length of the chain bridging two pyridinium rings
3) the presence of the oxime group
4) the position of the oxime group at the pyridinium ring
5) the number of the oxime groups

Generally, bisquaternary oximes have higher affinity toward both intact and inhibited AChE, therefore higher potency to reactivate nerve agent inhibited AChE compared to monoquaternary oximes. (9)

The reactivators with the oxime group in position four can reactivate tabun inhibited AChE, while those having the oxime group in position two are efficient reactivators of cyclosarin inhibited AChE. Therefore the oxime HI-6 cannot reactivate tabun inhibited AChE. (9)

On the other hand number of the oxime groups is not so important. K-203 has only one oxime group and is more effective in the reactivation of tabun inhibited AChE than bispyridinium oximes, such as obidoxime (9) (Kassa J, Karasova J, Musilek K, Kuca K, Jung Y-S 2008). The oxime HI-6 has only one oxime group and is significantly more effective in reactivating soman and cyclosarin inhibited AChE than bispyridinium oximes, such as obidoxime, methoxime and tromedoxime. (25)

The chain linking two quaternary nitrogen heterocycles in bispyridinium oximes presents influence on the dephosphylation and thus reactivation process. The tri and tetracarbon chain seems to be the most suitable for the efficient reactivation soman, tabun or cyclosarin inhibited AChE. (9, 25)

3.3.2. Pyridinium oximes

Oximes used in clinical application are divided in two groups: the monopyridinium and bispyridinium oximes. Currently only one monopyridinium oxime (pralidoxime) is available, while bispyridinium class includes several substances (trimedoxime, obidoxime, HI-6 and HLö-7).

3.3.2.1. Pralidoxime (PAM-2)

Pralidoxime (2-[(hydroxyimino)methyl]-1-methylpyridinium-bromide *(VIII)*, has been firstly synthesized in the USA in 1955. PAM-2 is sufficient in reactivating AChE inhibited with sarin or VX. It is not efficient in the reactivation of tabun or soman inhibited enzyme. (3)

PAM-2 is a quaternary pyridinium salt and does not penetrate the blood brain barrier Therefore a pro-drug, pro-2-PAM, was synthesized to pass into the central nervous system. In contrast to the expectation, it turned out to be less effective than PAM-2 against poisoning with paraoxon. However, it appears that if given with atropine, PAM-2 can cross the blood brain barrier at higher concentrations. The use of PAM-2 iodide with atropine and diazepam was effective in the treatment of Tokyo sarin attack in 1995. However, this oxime should not be a drug of choice in the nerve agent poisoning because it lacks efficacy against tabun and soman. (3)

There is a possibility to improve pralidoxime by changing its side chain on the pyridinium nitrogen. Various aliphatic and aromatic side chains were used to improve the properties of pralidoxime, such as its lipophilicity and reactivation ability. Pralidoxime is more lipophilic compared to bisquaternary compounds and it penetrates through blood-brain barrier in 10%. Regarding to its low reactivation ability, it still reactivates AChE in the brain tissue, where the bisquaternary compounds penetrate

only in 6% but with better reactivation ability. Furthermore, the allyl and aryl-alkyl side chains were designed not only for the increasing the lipophilicity, but also for the presence of π-electrons. The π-electrons suppose to interact with the AChE active site via π-π or cation-π interactions. The modified chain had an extensive impact on reactivation capability of monoquaternary compounds. (26)

3.3.2.2. Trimedoxime (TMB-4)

Trimedoxime (1,1'-propane-1,3-diylbis{4-[(hydroxyimino)methyl]pyridinium}-dibromide) *(IX)* was investigated in the USA, 1957. It is the only bispyridinium oxime with a propylene bridge between the two pyridinium rings. TMB-4 is more potent than PAM-2 in the reactivation of the DFP inhibited AChE and than LüH-6 in the case of the tabun inhibited enzyme. TMB-4 was the first efficient oxime in the treatment of intoxication with tabun. It can also protect poisoning with sarin or VX, but not with soman. However, it was shown that TMB-4 was the most toxic oxime among PAM-2, LüH-6 and HI-6. (3)

3.3.2.3. Obidoxime (LüH-6)

LüH-6 1,1′-(oxydimethanediyl)bis{4-[(hydroxyimino)methyl]pyridinium}-dibromide,*(X)* was named after scientists A. Lüttringhaus and I. Hagedorn who studied this oxime in Germany in 1964. It proved a significant potential as an antidote in toxication with OPC. LüH-6 given with atropine efficiently protected against poisoning with tabun, sarin and VX. It was even more effective than TMB-4 as an antidote against poisoning with tabun. Same as PAM-2 and TMB-4, LüH-6 was unsuccessful in soman intoxication in mice, pigs and primates. Obidoxime is also not able to sufficiently protect cyclosarin exposed people from toxic effects. However, this oxime shows the hepatotoxic nature. (3)

3.3.2.4. Asoxime (HI-6)

The first oxime reactivating soman inhibited AChE was synthesized in 1966. It was called HS-6 in honour of K. Schoene and I. Hagedorn. Thereafter its analogue was synthesized by I. Hagedorn and named HI-6 after her initials. HI-6 (4-carbamoyl-1-[({2-[(hydroxyimino)methyl]pyridinium-1-yl}methoxy)methyl]-dibromidewas shown to be more efficient than HS-6 and LüH-6 in protection from soman, sarin, cyclosarin and especially VX intoxication. On the other hand, HI-6 cannot reactivate tabun inhibited AChE. The toxicity of HI-6 is low, the lowest among the other oximes, therefore it can be administered at higher doses than other commonly used oximes. (3)

HI-6 dimethanesulphonate was shown to have the same pharmacokinetics as HI-6 dichloride salt. HI-6 dichloride may be limited by factors such as poor solubility or instability in aqeous media. HI-6 dimethanesulphonate was described as more soluble and stable salt than dichloride. The higher solubility and stability of HI-6 dimethansulphonate makes it possible to increase the dose and thus its effectiveness in the antidotal treatment of poisoning of sarin, soman and cyclosarin. Therefore the replacement of dichloride anion by dimethansulphonate anion in the oxime HI-6 may be useful for treating nerve agent poisoning. (27)

3.3.2.5. HLö-7

The fourth Hagedorn's oxime (after LüH-6, HS-6 and HI-6) was HLö-7 1-[({2,4-bis[(hydroxyimino)methyl]pyridinium-1-yl}methoxy)methyl]-4-carbamoylpyridinium-dibromide. It was investigated in Germany in 1986 and was named after I. Hagedorn and M. Löffler. HLö-7 induces a significant reactivation of AChE in mice inhibited with tabun, sarin, soman and cyclosarin. Both HLö-7 and HI-6 can antagonize sarin induced hypothermia; the toxicity of HLö-7 is higher than that of HI-6 in pigs. HLö-7 turned out to be more effective than HI-6 against tabun and VX intoxication and less effective against soman, sarin and cyclosarin poisoning. HLö-7 showed significantly efficient protection against tabun poisoning compared to HI-6, while HI-6 was only slightly better than HLö-7 in soman intoxication. (3)

To determine the best oxime against tabun, soman, sarin and cyclosarin intoxication these criteria are applied (3):

1) Ability to reactivate AChE inhibited by the nerve agents *in vitro* and *in vivo*

Only HLö-7 can reactivate AChE inhibited by tabun, sarin, soman and cyclosarin *in vitro* and *in vivo*. It is trailed by HI-6, which could not reactivate tabun inhibited enzyme.

2) Ability to provide survival of animals with lethal doses of the nerve agents given with atropine

Both HLö-7 and HI-6 administred with atropine provide *in vivo* protection against the poisoning of the five nerve agents, with HI-6 more efficient in case of soman, sarin and cyclosarin, and less effective in case of tabun and VX intoxication.

3) Ability to access the blood brain barrier and central nervous system at significant and relevant concentrations when given with atropine

Although no oxime studied in animals without carbamate pretreatment can induce reactivation of AChE inhibited by soman, only HLö-7 and HI-6 can reactivate AChE inhibited by sarin and antagonize sarin induced hypothermia, prove the ability of these oximes to pass the brain at relevant concentrations to exert their central antidotal effects.

4) Ability to imply some pharmacological effects such as influence on the release of acetylcholine from presynaptic nerve endings

All oximes have shown some pharmacological effects. It appears that HI-6 and HLö-7 have some prejunctional features and decrease the amount of acetylcholine released into the synaptic cleft in AChE inhibition by soman.

5) Toxicity of the oxime *per se*

Among presently used oximes HI-6 is by far the least toxic oxime, followed by HLö-7, PAM-2, LüH-6 and TMB-4.

6) Stability of the oxime

The older oximes PAM-2, TMB-4 and LüH-6 are stable in water solutions and can be prepared in a form of ampoules. HI-6 and HLö-7 are not stable in water, therefore they are kept for longer time in the form of powder that has to be dissolved immediately before the administration.

7) Ability for formulation of the oxime in an auto-injector

HI-6 and HLö-7 were prepared in the form of the wet-dry auto-injector. Similar systems containing PAM-2, TMB-4 and LüH-6 also exist, but only in the solution with atropine.

3.3.2.6. K-oximes

In a search of superior oximes, the K-reactivators were developed by Kuca and Musilek at the Department of Toxicology at the Faculty of Military Health Sciences (University of Defense) in Hradec Kralove in the Czech Republic. The newly developed oximes are bisquaternary symmetric (**K-33**, **K-74**, **K-75**, **K-107**, **K-108**, **K-113** and **K-114**) and asymmetric (BI-6, **K-27**, **K-48** and **K-53**) pyridinium aldoximes with the aldoxime group at the position two (BI-6, **K-27**, **K-48** and **K-53**) and four (**K-27**, **K-48**, **K-74**, **K-75**, **K-113** and **K-114**) of the pyridinium rings. All symmetrical compounds have two aldoxime groups and most asymmetrical ones only one. Some are asymmetrically substituted such as **K-53** that has two aldoximes groups at positions two and four of the pyridine rings (20).

K-27 [3-(4-carbamoylpyridinium)-1-{4-(hydroxyiminomethylpyridinium}- propane dibromide and **K-48** [4-(4-carbamoylpyridinium -1-(4-hydroxyiminomethylpyridinium) butane dibromide] present two bisquaternary asymmetric pyridinium aldoximes with only one aldoxime group in position four of the pyridinium ring were the first K-oximes developed. Some studies demonstrated their ability to protect AChE from inhibition by paraoxon, methyl paraoxon, tabun and VX, which makes theses oximes promising substances for the treatment of OPC poisoning (21). In the case of **K-27** the reactivation potency of tabun inhibited AChE is higher than that of the oxime HI-6. HI-6 is currently regarded to be the promising reactivator of organophosphate inhibited AChE (24). Also **K-27** and **K-48** oximes are able to reactivate AChE inhibited after malathion exposure (23).

The compounds with two aldoxime groups are another type of K-substances. They are either symmetrical (**K-005**, **K-33**, **K-74** and **K-75**) or non-symmetrical (**K-53**, 1-[(2-(hydroxyiminomethyl)pyridinium]-4-[(4-hydroxyiminomethyl)pyridinium]but-2-ene dibromide).

K-005 [1,3-bis[(2-(hydroxyiminomethyl)pyridinium]propane dibromide] is able to reactivate cyclosarin and VX inhibited AChE, however its reactivation potency is lower than that of pralidoxime, obidoxime and HI-6. On the other hand **K-005** does not reactivate sarin inhibited AChE. In the case of tabun poisonings, its reactivation is lower as compared to HI-6 and comparable with pralidoxime and obidoxime (24).

K-33 [1,4-bis[(2-(hydroxyiminomethyl)pyridinium]butane dibromide] was found to be more efficacious reactivator of sarin and cyclosarin inhibited AChE than pralidoxime or obidoxime, but it did not reach the effectiveness of the oxime HI-6. On the other hand **K-33** is more successful than HI-6 to reactivate tabun inhibited AChE (24).

K-74 [1,4-bis[4-(hydroxyiminomethyl)pyridinium]butane dibromide] and **K-75** [1,4-bis[4-(hydroxyiminomethyl)pyridinium)but-2-en dibromide] were effective in reactivating tabun inhibited AChE (21).

Most recently a group of K-oximes containing a xylene linker has been developed. These bisquaternary symmetric pyridinium aldoximes with the functional aldoxime at position two (**K-107**, **K-108**) or four (**K-113** and **K-114)** are successful reactivators of AChE inhibited by tabun, cyclosarin and paraoxon (21).

K-53, **K-74**, **K-75**, **K-107**, **K-108**, **K-113** and **K-114** oximes showed promising *in vitro* reactivation capacity of AChE inhibited by DFP (20). However, when considering the application of new oximes *in vivo,* **K-27** showed the best mortality reducing effect, which is significantly superior to that of **2-PAM**, **K-48**, **K-74**, **K-107**, **K-108** and **K-113**. Unfortunately **K-107**, **K-108** and **K-113** (which strongly inhibit AChE *in vitro*) are *in vivo* more toxic than all the other oximes, therefore can be only administered at a low dosage, which is insufficient to protect from DFP induced mortality (21).

Newly developed oximes **K-74** and **K-75** can be considered to be promising oximes for the antidotal treatment of tabun poisonings. Due to their neuroprotective effects, they appear to be more suitable oximes for the antidotal treatment of acute tabun poisonings than the oxime HI-6. In addition, **K-75** seems to be slightly more effective than **K-74** after tabun poisoning. **K-75** is promising oxime for antidotal treatment of tabun poisoning although its neuroprotective potency does not markedly differ from the potency of obidoxime. Both of them are suitable oximes for the reactivation of VX induced poisoning. They are not able to reactivate cyclosarin inhibited AChE (25).

The potency of oximes **K-117** [1,5-bis[4-(hydroxyiminomethyl)pyridinium]-3-oxapentane dibromide] and **K-127** [5-(4-carbamoylpyridinium)-1-[4-(hydroxyiminomethyl)pyridinium] -3-oxapentane dibromide] in reactivation tabun inhibited AChE did not prevail over the effectiveness of currently available obidoxime and trimedoxime. Therefore they are not suitable for the replacement of commonly used oximes for the treatment of tabun poisoning (9).

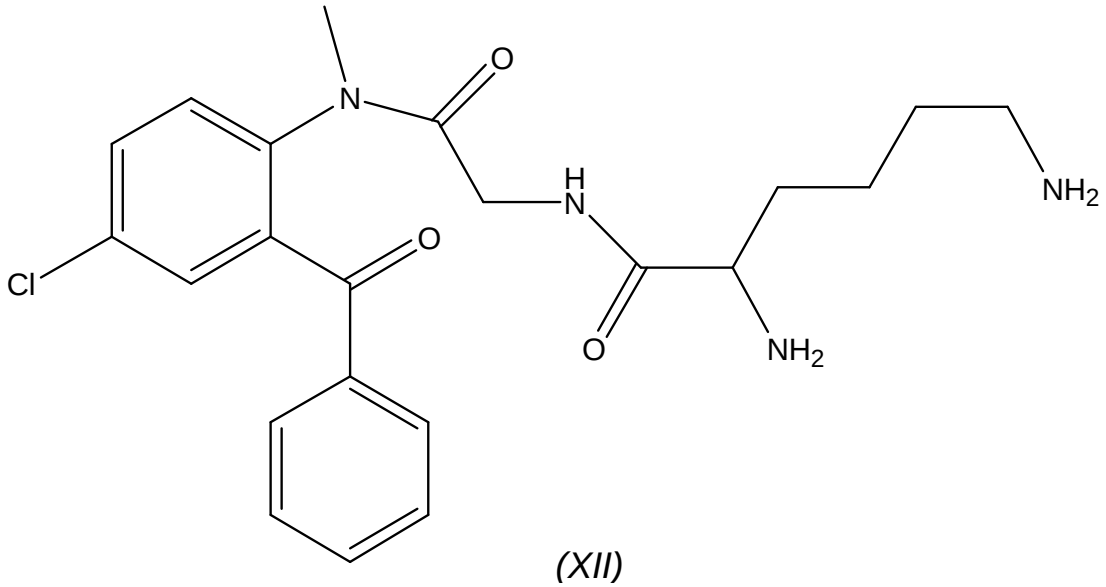
K-203 [(*E*)-1-(4-carbamoylpyridinium)-4-[4-(hydroxyiminomethyl)pyridinium)but-2-ene dibromide] seems to be significantly more effective to reactivate tabun inhibited AChE and to reduce toxic effects of tabun than all currently used oximes. Therefore, **K-203** is suitable for the replacement of commonly used oximes for the treatment of tabun poisoning (13).

K-206 [(*E*)-1-(3-carbamoylpyridinium)-4-[4-(hydroxyiminomethyl)pyridinium)but-2-ene dibromide] and **K-269** [(*E*)-1-[4-(N´-hydroxycarbamimidoyl)pyridinium]-4-[4-(hydroxyiminomethyl)pyridinium]but-2-ene dibromide] are as effective in the reactivation of cyclosarin inhibited AChE as obidoxime, but because their reactivating potency is significantly lower than that of HI-6, they are not suitable replacements for the currently available oximes for the treatment of cyclosarin intoxication (28).

3.3.3. Auto-injectors for application of antidotes used in OPC poisoning

Some countries have been developing auto-injectors for the protection of their armed forces. They use pyridostigmine as a pretreatment antidote and atropine as an antimuscarinic drug. And they also have an oxime as a part of the antidotal combination. The choice of the oxime differs very much, from PAM-2 salts in the UK and the US, to LüH-6 in Norway, Finland, Germany and Holland and HI-6 in Sweden and Canada. (3)

Some of these countries have an anticonvulsant in a form of auto-injector. The advantage of avizafone (prodrug of diazepam; *XII*) over diazepam is it's hydrosolubility, that allows its presence in the water solutions of atropine as a wet part of the wet-dry auto-injectors, with HI-6 powder as the dry part. (3)



(XII)

Many armies have auto-injectors against nerve agents containing atropine and one oxime. However, some problems include the lack of a commercial source for the supply of HI-6, unpractical system of multiple auto-injectors, and incomplete data package to support a regulatory submission. (3)

HI-6 seems to be the best antidote against poisonings with nerve agents versus its analogue HLö-7. Although HLö-7 has few advantages over HI-6, it is obvious that both oximes are superior to PAM-2, TMB-4 and LüH-6 in the treatment of intoxications with nerve agents given with atropine. (3)

Table 2 **AUTO-INJECTOR CONTENTS OF SOME COUNTRIES (3)**

Country	Prophylaxis	Anticholinergic	Oxime	Anticonvulsant
United Kingdom	pyridostigmine	atropine	P2S	avizafone
United States	pyridostigmine	atropine	PAM-2	diazepam
Norway	pyridostigmine	atropine	LüH-6	diazepam
Finland	pyridostigmine	atropine	LüH-6	-
Germany	pyridostigmine	atropine	LüH-6	-
Holland	pyridostigmine	atropine	LüH-6	avizafone
Sweden	pyridostigmine	atropine	HI-6	-
Canada	pyridostigmine	atropine	HI-6	diazepam

Pyridostigmine is a carbamate inhibitor of cholinesterases. Carbamates confer some protection from the lethal effects of some OPC. Oral pyridostigmine for pre-exposure treatment of some nerve agents should be administered one 30 mg tablet every 8 hours. The concept is to block the cholinesterase reversibly using the less potent reversible inhibitor (carbamate) to deny access to the active site of the enzyme to the more potent irreversible organophosphorus inhibitor (nerve agent) on subsequent exposure. The combined use of carbamate pre-treatment followed with atropine, oxime and benzodiazepine (diazepam) has been initially advocated by the British. Pyridostigmine

alone is considered to be unsufficient, only when followed with atropine and oxime is supposed to be effective. (29)

Anticholinergic drug block effects of overstimulation by accumulated acetylcholine at muscarinic receptor sites. (30)

Oximes repair biochemical lesions by dephosphylating the enzyme and restoring activity. (30)

Generally, the efficacy of AChE reactivators depends on their affinity and reactivity for OPC inhibited enzyme, which in turn is dependent on the structure. The mechanism of the reactivation effect is based on the nucleophilic activity of oxime anion bounded on the pyridinium ring. Hence this part of the molecule is essential for the activity and can be regarded as the pharmacophore. The structural modifications in other parts of the molecule, such as number and position of hydroxyiminomethyl groups on the pyridinium ring, the length and character of linking chain and presence of additional substituents can result in changes of affinity and pharmacokinetic properties, which can finally result in a broad spectrum reactivator with low instrinsic toxicity. (30)

3.4. LINKERS BETWEEN TWO PYRIDINIUM RINGS

Development of new oxime reactivators continues by designing the substances that differ from the currently used oximes in the length and atoms connecting chain between two pyridinium rings. (31)

Research is interested in oxygen atom, because oxime reactivators having the linkers of oxygen atom and methylene such as HI-6 show stronger reactivation activity compared to other available reactivators such as obidoxime and pralidoxime. New oximes with a longer ether linker connecting two pyridine rings were synthesized. The reactivation potency of compounds in which two -pyridiniumaldoximes were connected with $CH_2O(CH_2)_nOCH_2$ linkers between two quaternary nitrogens was evaluated to reactivate AChE inhibited with VX agent. Derivative with $CH_2O(CH_2)_2OCH_2$ linker was able to reactivate inhibited AChE by organophosphorus agent VX, therefore it may provide a useful therapeutic potential for the reactivation of AChE inhibited by VX. (31)

The reactivators with (*E*)-but-2-ene linker were synthesized in an effort to extend the properties of the oxime K-203 [4-(4-carbamoylpyridinium)-1-[4-(hydroxyiminomethyl)-pyridinium]but-2-ene dibromide].. The reactivation was tested on tabun and paraoxon inhibited AChE.

For tabun inhibited AChE at least one oxime in position four on the heteroaromatic ring is needed for the reactivation, whilst an oxime in position two has a low or no reactivation. The optimal linker length for tabun intoxication varies from three to four carbon-carbon bonds. The (*E*)-but-2-ene linker is slightly longer than three, but slightly shorter than four carbon-carbon bonds due the presence of double bond, which restricts the conformational flexibility of (*E*)-but-2-ene linker. Also the (*E*)-but-2-ene linker was more effective than (*Z*)-but-2-ene linker. The influence of the second heteroatomic ring or its substitution for tabun intoxication was established. The replacement of the second pyridinium ring by another heteroatomic ring such as, pyridazinium, quinolinium,

isoquinolinium decreased the reactivation ability to zero levels. Derivatives with lipophilic substituents on pyridinium ring, such as methyl, *tert*-butyl, phenyl and benzyl, showed minimal reactivation of tabun inhibited AChE. Some hydrophilic substituents, such as carboxyl and resulted in a decreased but still notable reactivation ability. The mechanism of the interaction of the hydrophilic sustituents with inhibited enzyme is not clear, weak hydrogen bond interactions are predicted.

For paraoxon inhibited AChE at least one oxime group in position four is beneficial compared to an oxime in position two. The reactivation ability of monooxime compounds for paraoxon showed less changes in the enzyme's active site compared to tabun inhibited AChE, probably due to paraoxon's slower aging process. Suprisingly, for mooximes the differences among other heteroatomic rings, lipophilic and hydrophilic substituents were not as significant as in the case of tabun. The isoquinolinium monooxime reactivator was the best compound from the heteroatomic rings. It is not clear if weak hydrophobic or hydrophilic interactions play a role in the reactivation ability paraoxon inhibited AChE.

The results of this study showed interesting differences between reactivation of tabun and paraoxon. While substances with hydrophilic substitution on monoxime pyridinium ring were efficient in reactivation of tabun intoxication, reactivators with both hydrophilic and lipophilic substituents and other heterocyclic moieties in the molecule were able to reactivate paraoxon inhibited AChE. (32)

The flexibility of aldoximes has been confirmed as an important factor affecting the interactions. It seems that the introduction of a double bond in the linker between aldoxime's two pyridinium rings has a strong influence in AChE reactivation. The reactivation of tabun inhibited AChE by K-74 and K-75 oximes was very efficient, more than by classical aldoxime TMB-4 and oxime K-48. However, K-74 and K-75 assisted reactivation did not completely restore the enzyme activity. This could be a sign of postinhibitory dealkylation of phosphorylated enzyme or the reinhibition of reactivated

enzyme by the phosphorylated aldoximes. The oxime K-114, most rigid one, showed the lowest reactivation ability. Limited flexibility and additional interactions with aromatic ring in the linker did not allow the right orientation for nucleophilic attack on phosphorylated serine. (33)

4. EXPERIMENTAL

4.1. SYNTHESIS

4.1.1. General

All solvents used (acetone, acetonitrile, dimethylformamide, ethyl acetate) were supplied by FLUKA and SIGMA-ALDRICH in a sufficient quality. The reaction progress and determination of any impurities present was monitored on TLC (DC – Alufolien Cellulose F, Merck, Germany. Butanol – acetic acid– water (5:1:2) was used as a mobile phase, Dragendorff reagent was used for detection.

NMR Spectrometry was done on Varian Gemini 300 (^{1}H 300 MHz, ^{13}C 75 MHz, Palo Alto CA, USA). Chemical shifts for ^{1}H and ^{13}C are given in ppm (δ), signals are designated as s (singlet), d (doublet) t (triplet) and m (multiplet).

4.1.2. **Preparation of monoquaternary substances**

1-FZ-1

1-methyl-4-(*N*′-hydroxycarbamimidoyl)pyridinium iodide

N′-hydroxypyridine-4-carboximidamide (0.3 g; 2.2 mmol) and 50 ml of acetonitrile were heated until totally dissolved. Afterwards iodomethane (0.62 g; 4.4 mmol) was added. The reaction time was 12 hours at 40 ˚C. Cooled down to laboratory temperature, filtrated and recrystalized from MeCN. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 279.08

Yield: 49 %.

M.p.: 203 – 204 °C

^{1}H NMR (300 MHz, DMSO d6): δ (ppm) 10.92 (s, 1H, =C=NOH), 8.95 (d, 2H, J = 6.8 Hz, H-2,6), 8.26 (d, 2H, J = 6.7Hz, H–3,5), 6.42 (s, 2H, -NH_2), 4.31 (s, 3H, -CH_3)
^{13}C NMR (75 MHz, DMSO d6): δ (ppm) 147.62, 147.22, 145.55, 122.61 , 47.64

1-FZ-5

1-(4-bromobut-2-en-1-yl)-4-(*N*'-hydroxycarbamimidoyl)pyridinium bromide

N'-hydroxypyridine-4-carboximidamide (2 g; 1.64 mmol) was dissolved in 60 ml of acetone. Afterwards 1,3-dibromoprop-1-ene (17.51 g; 8.19 mmol) was added. The reaction time was 1.5 hours at 60 ˚C. Cooled down to laboratory temperature and filtrated. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 336.02

Yield: 96 %.

M.p.: 185 – 188 °C

^{1}H NMR (300 MHz DMSO d6) δ (ppm) 12.85 (s, 1H, =C=NOH), 9.02 (d, 2H, *J* = 6.54 Hz, H-2,6), 8.44 (s, 1H), 8.27 (d, 2H, *J* = 6.54 Hz, H-3,5), 6.30 (s, 2H, -NH_2), 5.31 (d, 4H, *J* = 4.80Hz, -CH=CH-), 4.18 (s, 2H, CH_2)
^{13}C NMR (75 MHz, DMSO d6) δ (ppm) 148.67, 145.06, 144.85, 133.70, 127.69, 124.18, 60.27, 32.00

1-FZ-15

1-ethyl-4-(*N'*-hydroxycarbamimidoyl)pyridinium iodide

N'-hydroxypyridine-4-carboximidamide (0.2 g; 1.46 mmol) and 50 ml of MeCN were heated together until totally dissolved. Afterwards iodoethane (0.341 g; 2.19 mmol) was added. The reaction time was 21 hours at 70 ˚C. Cooled down to laboratory temperature, left in the fridge for several months, filtrated and purified in EtOAc. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 293.10

Yield: 51 %.

M.p.: 198 – 200 °C

^{1}H NMR (300 MHz, DMSO d6) δ (ppm) 10.94 (s, 1H, =C=NOH), 9.07 (d, 2H, *J* = 6.03 Hz, H - 2,6), 8.30 (d, 2H, *J* = 6.00 Hz, H – 3,5), 6.44 (s, 2H, -NH_2), 4.60 (t, *J* = 4.76 Hz, N-CH_2), 1.53 (t, 3H, *J* = 7.05 Hz, N-CH_2-CH_3),
^{13}C NMR (75 MHz, DMSO d6) δ (ppm) 147.65, 146.93, 144.25, 122.66, 55.68, 16.05

1-FZ-16

1-propyl-4-(*N*'-hydroxycarbamimidoyl)pyridinium iodide

N'-hydroxypyridine-4-carboximidamide (0.2 g; 1.46 mmol) and 10 ml of MeCN were heated together until totally dissolved. Afterwards 1-iodopropane (0.372 g; 2.19 mmol) was added. The reaction time was 21 hours at 70 ˚C. Cooled down to laboratory temperature, left in the fridge for several months, filtrated and purified in EtOAc. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 307.13

Yield: 44 %.

M.p.: 202 – 204 °C

^{1}H NMR (300 MHz, DMSO d6) δ (ppm) 10.96 (s, 1H, =C=NOH) , 9.05 (d, 2H, *J* = 6.74 Hz, H - 2,6), 8.30 (d, 2H, *J* = 6.73 Hz, H – 3,5), 6.44 (s, 2H, -NH_2), 4.53 (t, 2H *J* = 7.22 Hz, N-CH_2), 1.93 (m, 2H, N-CH_2-*CH_2*), 0.88 (t, 3H, *J* = 7.34 Hz, N-CH_2-CH_2-*CH_3*)
^{13}C NMR (75 MHz, DMSO d6) δ (ppm) 147.21, 147.19, 144.77, 144.72, 122.95, 61.68, 24.14, 10.41

1-FZ-35

1-pentyl- 4-(*N*'-hydroxycarbamimidoyl)pyridinium bromide

N'-hydroxypyridine-4-carboximidamide (0.5 g; 3.65 mmol) and 50 ml of MeCN were heated together until totally dissolved. Afterwards 1-bromopentane (0.83 g; 5.49 mmol) was added. The reaction time was 48.5 hours at 70 ˚C. Cooled down to laboratory temperature, left in the fridge overnight, filtrated. Recrystallized from MeCN and purified in EtOAc. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 288.19

Yield: 12 %.

M.p.: 203 – 205 °C

^{1}H NMR (300 MHz, DMSO d6) δ (ppm) 10.95 (s, 1H, =C=NOH), 9.08 (d, 2H, *J* = 7.03 Hz, H - 2,6), 8.31 (d, 2H, *J* = 6.98 Hz, H – 3,5), 6.44 (s, 2H, -NH_2), 4.57 (t, *J* = 7.38, Hz, N-CH_2), 2.03 (m, 2H, N-CH_2-*CH_2*), 1.37 (m, 4H, N-CH_2-CH_2-*CH_2*), 0.86 (t, 3H, *J* = 7.01, 7.01 Hz, N-CH_2-CH_2-CH_2-*CH_3*)

^{13}C NMR (75 MHz, DMSO d6) δ (ppm) 147.70, 146.95, 144.45, 122.69, 60.02, 30.14, 27.42, 21.47, 13.65

1-FZ-30

1-nonyl-4-(*N*'-hydroxycarbamimidoyl)pyridinium bromide

N'-hydroxypyridine-4-carboximidamide (0.5 g; 3.65 mmol) and 50 ml of MeCN were heated together until totally dissolved. Afterwards 1-bromononane (1.13 g; 5.45 mmol) was added. The reaction time was 59.5 hours at 70 ˚C. Cooled down to laboratory temperature, filtrated. Recrystallized from MeCN. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 344.30

Yield: 24 %.

M.p.: 194 – 196 °C

^{1}H NMR (300 MHz, DMSO d6) δ (ppm) 10.95 (s, 1H, =C=NOH), 9.09 (d, 2H, *J* = 6.92 Hz, H - 2,6), 8.31 (d, 2H, *J* = 6.90 Hz, H – 3,5), 6.44 (s, 2H, -NH_2), 4.57 (t, 2H, *J* = 7.31, Hz, N-CH_2), 1.91 (m, 2H, N-CH_2-*CH_2*), 1.24 (m, 12H, N-CH_2-CH_2-*CH_2*), 0.84 (t, 3H, *J* = 6.28 Hz, N-CH_2-CH_2-CH_2-*CH_3*)

^{13}C NMR (75 MHz, DMSO d6) δ (ppm) 147.70, 146.95, 144.47, 122.69, 60.01, 31.14, 30.46, 28.65, 28.48, 28.31, 25.29, 21.99

1-FZ-29

1-decyl-4-(*N*'-hydroxycarbamimidoyl)pyridinium bromide

N'-hydroxypyridine-4-carboximidamide (0.5 g; 3.65 mmol) and 50 ml of MeCN were heated together until totally dissolved. Afterwards 1-bromodecane (1.21 g; 5.47 mmol) was added. The reaction time was 45 hours at 70 ˚C. Cooled down to laboratory temperature, left in the fridge overnight, filtrated. Recrystallized from MeCN and purified in EtOAc. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 358.33

Yield: 50 %.

M.p.: 199 – 201 °C

1H NMR (300 MHz, DMSO d6) δ (ppm) 10.95 (s, 1H, =C=NOH), 9.08 (d, 2H, *J* = 6.93 Hz, H - 2,6), 8.31 (d, 2H, *J* = 6.91 Hz, H – 3,5), 6.45 (s, 2H, -NH_2), 4.56 (t, 2H, *J* = 7.33, Hz, N-CH_2), 1.91 (m, 2H, N-CH_2-*CH_2*), 1.24 (m,14H, N-CH_2-CH_2-*CH_2*), 0.84 (t, 3H, *J* = 6.66 Hz, N-CH_2-CH_2-CH_2-*CH_3*)

^{13}C NMR (75 MHz, DMSO d6) δ (ppm) 146.94, 144.46, 122.68, 60.05, 31.19, 30.46, 28.78, 28.70, 28.57, 28.31, 25.30, 22.02, 13.89

1-FZ-28

1-dodecyl-4-(*N*'-hydroxycarbamimidoyl)pyridinium bromide

N'-hydroxypyridine-4-carboximidamide (0.5 g; 3.65 mmol) and 50 ml of MeCN were heated together until totally dissolved. Afterwards 1-bromododecane (1.36 g; 5.46 mmol) was added. The reaction time was 47 hours at 70 ˚C. Cooled down to laboratory temperature, left in the fridge overnight, filtrated. Recrystallized from MeCN. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 386.38

Yield: 19 %.

M.p.: 202 – 204 °C

^{1}H NMR (300 MHz, DMSO d6) δ (ppm) 10.96 (s, 1H, =C=NOH), 9.08 (d, 2H, *J* = 6.59 Hz, H - 2,6), 8.31 (d, 2H, *J* = 6.53 Hz, H – 3,5), 6.45 (s, 2H, -NH_2), 4.56 (t, 2H, *J* = 7.29, Hz, N-CH_2), 1.91 (m, 2H, N-CH_2-*CH_2*), 1.24 (m,18H, N-CH_2-CH_2-*CH_2*), 0.84 (t, 3H, *J* = 6.56 Hz, N-CH_2-CH_2-CH_2-*CH_3*)

^{13}C NMR (75 MHz, DMSO d6) δ (ppm) 149.40, 147.70, 146.94, 144.46, 122.69,119.59, 31.22, 28.89, 28.68, 28.33, 25.31, 22.03, 13.90

1-FZ-34

1-tetradecyl-4-(*N*'-hydroxycarbamimidoyl)pyridinium bromide

N'-hydroxypyridine-4-carboximidamide (0.5 g; 3.65 mmol) and 50 ml of MeCN were heated together until totally dissolved. Afterwards 1-bromotetradecane (1.52 g; 5.48 mmol) was added. The reaction time was 45 hours at 70 ˚C. Cooled down to laboratory temperature, filtrated and purified in EtOAc. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 414.44

Yield: 24 %.

M.p.: 205 – 207 °C

^{1}H NMR (300 MHz, DMSO d6) δ (ppm) 10.95 (s, 1H, =C=NOH), 9.08 (d, 2H, *J* = 7.04 Hz, H - 2,6), 8.31 (d, 2H, *J* = 6.98 Hz, H – 3,5), 6.44 (s, 2H, -NH_2), 4.56 (t, 2H, *J* = 7.36, Hz, N-CH_2), 1.91 (m, 2H, N-CH_2-*CH_2*), 1.26 (m,22H, N-CH_2-CH_2-*CH_2*), 0.90 (t, 3H, *J* = 6.53 Hz, N-CH_2-CH_2-CH_2-*CH_3*)

^{13}C NMR (75 MHz, DMSO d6) δ (ppm) 147.67, 146.91, 146.88, 144.48, 122.678, 60.4, 31.20, 30.45, 28.96, 28.94, 28.91, 28.61, 28.31, 25.23,

1-FZ-27

1-octadecyl-4-(*N*'-hydroxycarbamimidoyl)pyridinium bromide

N'-hydroxypyridine-4-carboximidamide (0.5 g; 3.65 mmol) and 50 ml of MeCN were heated together until totally dissolved. Afterwards 1-bromooctandecane (1.82 g; 5.47 mM) was added. The reaction time was 43 hours at 70 ˚C. Quick filtration while still hot. Recrystallized from MeCN and purified in EtOAc. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 470.34

Yield: 16%.

M.p.: 184 – 185 °C

1H NMR (300 MHz, DMSO d6) δ (ppm) 10.98 (s, 1H, =C=NOH), 9.08 (d, 2H, *J* = 6.85 Hz, H - 2,6), 8.31 (d,2H, *J* = 6.83 Hz, H – 3,5), 6.44 (s, 2H, -NH_2), 4.56 (t, 2H, *J* = 7.24, Hz, N-CH_2), 1.90 (m, 2H, N-CH_2-*CH_2*), 1.22 (m, 30H, N-CH_2-CH_2-*CH_2*), 0.84 (t, 3H *J* = 6.55 Hz, N-CH_2-CH_2-CH_2-*CH_3*)

^{13}C NMR (75 MHz, DMSO d6) δ (ppm) 147.70, 146.92, 144.43, 122.67, 60.03, 31.20, 30.46, 28.89, 28.66, 28.32, 25.31, 22.00, 13.86

4.2.1. **Preparation of bisquaternary substances**

1-FZ-7

1,1'-(ethane-1,2-diyl)bis(4-[*N*'-hydroxycarbamimidoyl)pyridinium] bromide

N'-hydroxypyridine-4-carboximidamide (0.5 g; 3.65 mM) was dissolved in 10 ml of DMF. Afterwards 1,2-dibromoethane (0.31 g; 1.64 mM) was added. The reaction time was 19 hours at 100 ˚C. Cooled down to laboratory temperature, left in the fridge overnight, filtrated and purified in MeCN. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 462.14

Yield: 3 %.

M.p.: degradation above 280`C.

^{1}H NMR (300 MHz, DMSO d6): δ (ppm) 11.05 (s, 2H, =C=NOH), 9.05 (d, 4H, *J* = 6.64 Hz, H – 2,2′, 6,6′), 8.35 (d, 4H, *J* = 6.63 Hz, H – 3,3′, 5,5′), 6.47 (s, 4H, -NH_2), 5.26 (t, 4H, J = 7.23 Hz, N-CH_2)

^{13}C NMR (75 MHz, , DMSO d6): δ (ppm) 148.53, 146.90 144.98, 122.90

1-FZ-4

1,1'-(butane-1,4-diyl)bis[4-(*N*'-hydroxycarbamimidoyl)pyridinium] bromide

N'-hydroxypyridine-4-carboximidamide (0.5 g; 3.65 mM) was dissolved in 10 ml of DMF. Afterwards 1,4-dibromobutane; (0.35 g; 1.64 mM) was added. The reaction time was 14 hours at 100 ˚C. Cooled down to laboratory temperature, left in the fridge overnight, filtrated and purified in MeCN. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 490.19

Yield: 60 %.

M.p.: 248 – 250 °C

^{1}H NMR (300 MHz, DMSO d6): δ (ppm) 10.96 (s, 2H, =C=NOH), 9.11 (d, 4H, *J* = 6.65 Hz, H – 2,2′, 6,6′), 8.32 (d, 4H, *J* = 6.58 Hz, H – 3,3′, 5,5′), 6.46 (s, 4H, -NH_2), 4.67 (t, 4H, *J* = 4.94 Hz, N-CH_2), 1.97 (m, 4H, N-CH_2-CH_2),
^{13}C NMR (75 MHz, DMSO d6): δ (ppm) 148.03, 147.19, 144.82, 122.97, 69.36, 27.15

1-FZ-6

1,1'-(pentane-1,5-diyl)bis[4-(*N*'-hydroxycarbamimidoyl)pyridinium] bromide

N'-hydroxypyridine-4-carboximidamide (0.5 g; 3.65 mM) was dissolved in 10 ml of DMF. Consequently 1,5-dibromopentane; (0.38 g; 1.65 mM) was added. The reaction time was 10 hours at 100 ˚C. Cooled down to laboratory temperature, left in the fridge overnight, filtrated and purified in MeCN. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 504.22

Yield: 74 %.

M.p.: 266 – 268 °C

^{1}H NMR (300 MHz, DMSO d6): δ (ppm) 10.96 (s, 2H, =C=NOH), 9.14 (d, 4H, *J* = 6.86 Hz, H – 2,2′, 6,6′), 8.33 (d, 4H, *J* = 6.84 Hz, H – 3,3′, 5,5′), 6.47 (s, 4H, -NH_2), 4.61 (t, 4H, *J* = 7.12 Hz, N-CH_2), 1.97 (m, 6H, N-CH_2-*CH_2*)
^{13}C NMR (75 MHz, DMSO d6): δ (ppm) 147.72, 146.94, 144.54, 122.69, 59.50, 29.70, 21.74

1-FZ-8

1,1'-(hexane-1,6-diyl)bis[4-(*N*'-hydroxycarbamimidoyl)pyridinium] bromide

N'-hydroxypyridine-4-carboximidamide (0.5 g; 3.65 mM) was dissolved in 10 ml of DMF. Afterwards 1,6-dibromohexane (0.40 g; 1.64 mM) was added. The reaction time was 14. 5 hours at 100 ˚C. Cooled down to laboratory temperature, left in the fridge overnight, filtrated and purified in MeCN. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 518.24

Yield: 61 %.

M.p.: 280 – 282 °C

^{1}H NMR (300 MHz, DMSO d6): δ ppm 10.95 (s, 2H, =C=NOH), 9.12 (d, 4H, *J* = 6.79 Hz, H – 2,2′, 6,6′), 8.32 (d, 4H, *J* = 6.77 Hz, H – 3,3′, 5,5′), 6.45 (s, 4H, -NH_2), 4.59 (t, 4H, *J* = 7.18 Hz, N-CH_2), 1.92 (m, 4H, N-CH_2-CH_2), 1.30 (m, 6H, N-CH_2-CH_2-CH_2)
^{13}C NMR (75 MHz, DMSO d6): δ (ppm) 147.72, 146.96, 144.50, 122.68, 59.80, 30.80, 24.62

1-FZ-9

1,1′-(heptane-1,7-diyl)bis[4-(*N*'-hydroxycarbamimidoyl)pyridinium] bromide

N′-hydroxypyridine-4-carboximidamide (0.5 g; 3.65 mM) was dissolved in 10 ml of DMF. Consequently 1,7-dibromoheptane (0.43 g; 1.64 mM) was added. The reaction time was 17 hours at 100 ˚C. Cooled down to laboratory temperature, left in the fridge overnight, filtrated and purified in MeCN and in acetone. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 532.27

Yield: 40 %.

M.p.: 230 – 232 °C

^{1}H NMR (300 MHz, DMSO d6): δ (ppm) 10.95 (s, 2H, =C=NOH), 9.11 (d, 4H, *J* = 5.61 Hz, H – 2,2′, 6,6′), 8.31 (d, 4H, *J* = 5.58 Hz, H – 3,3′, 5,5′), 6.45 (s, 4H, -NH_2), 4.58 (t, 4H, *J* = 6.92 Hz, N – CH_2), 1.91 (m, 4H, N-CH_2-*CH_2*), 1.29 (m, 6H, N-CH_2-CH_2-*CH_2*)
^{13}C NMR (75 MHz, DMSO d6): δ (ppm) 147.69, 146.95, 144.47, 122.69, 59.93, 30.31, 27.68, 25.03

1-FZ-10

1,1'-(octane-1,8-diyl)bis[4-(*N*'-hydroxycarbamimidoyl)pyridinium] bromide

N'-hydroxypyridine-4-carboximidamide (0.5 g; 3.65 mM) was dissolved in 10 ml of DMF. Afterwards 1,8-dibromooctane (0.45 g; 1.64 mM) was added. The reaction time was 13.5 hours at 100 ˚C. Cooled down to laboratory temperature, left in the fridge overnight, filtrated and purified in MeCN. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 546.30

Yield: 63 %.

M.p.: 270 – 272 °C

^{1}H NMR (300 MHz, DMSO d6) δ (ppm) 10.95 (s, 2H, =C=NOH), 9.11 (d, 4H, *J* = 6.08 Hz, H – 2,2′, 6,6′), 8.31 (d, 4H, *J* = 5.97 Hz, H – 3,3′, 5,5′), 6.45 (s, 4H, -NH_2), 4.58 (t, 2H, *J* = 7.12 Hz, N-CH_2), 1.90 (m, 4H, N-CH_2-CH_2), 1.28 (m, 8H, N-CH_2-CH_2-CH_2)
^{13}C NMR (75 MHz, DMSO d6) δ (ppm) 147.69, 146.95, 144.47, 122.70, 59.96, 30.42, 28.09, 25.21

1-FZ-11

1,1'-(nonane-1,9-diyl)bis[4-(*N*'-hydroxycarbamimidoyl)pyridinium]bromide

N'-hydroxypyridine-4-carboximidamide (0.5 g; 3.65 mM) was dissolved in 10 ml of DMF. Consequently 1,9-dibromononane (0.47 g; 1.64 mM) was added. The reaction time was 13 hours at 100 ˚C. Cooled down to laboratory temperature, left in the fridge overnight, filtrated and purified in MeCN and in acetone. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 560.32

Yield: 35 %.

M.p.: 272 – 274 °C

^{1}H NMR (300 MHz, DMSO d6) δ (ppm) 10.95 (s, 2H, =C=NOH), 9.12 (d, 4H, *J* = 5.88 Hz, H – 2,2′, 6,6′), 8.32 (d, 4H, *J* = 5.73 Hz, H – 3,3′, 5,5′), 6.45 (s, 4H, -NH_2), 4.58 (t, 2H, *J* = 7.15 Hz, N-CH_2), 1.89 (m, 4H, N-CH_2-CH_2), 1.26 (m, 10H, N-CH_2-CH_2-CH_2) ^{13}C NMR (75 MHz, DMSO d6) δ (ppm)147.94, 147.23, 144.74, 122.96, 60.26, 30.72, 28.73, 28.48, 25.53

1-FZ-12

1,1'-(decane-1,10-diyl)bis[4-(*N*'-hydroxycarbamimidoyl)pyridinium] bromide

N'-hydroxypyridine-4-carboximidamide (0.5 g; 3.65 mM) was dissolved in 10 ml of DMF. Afterwards 1,10-dibromodecane (0.49 g; 1.64 mM) was added. The reaction time was 22 hours at 100 ˚C. Cooled down to laboratory temperature, left in the fridge overnight, filtrated and purified in MeCN. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 574.35

Yield: 50 %.

M.p.: 280 – 282 °C

^{1}H NMR (300 MHz, DMSO d6) δ (ppm) 10.95 (s, 2H, =C=NOH), 9.10 (d, 4H, *J* = 6.80 Hz, H – 2,2′, 6,6′), 8.31 (d, 4H, *J* = 6.76 Hz, H – 3,3′, 5,5′), 6.45 (s, 4H, -NH_2), 4.57 (t, 2H, *J* = 7.15 Hz, N-CH_2), 1.89 (m, 4H, N-CH_2-CH_2), 1.25 (m, 12H, N-CH_2-CH_2CH_2) ^{13}C NMR (75 MHz, DMSO d6) δ (ppm) 147.69, 146.96, 144.47, 122.70, 60.03, 30.48, 28.60, 28.30, 25.31

1-FZ-13

1,1'-(undecane-1,11-diyl)bis[4-(*N*'-hydroxycarbamimidoyl)pyridinium] bromide

N'-hydroxypyridine-4-carboximidamide; (0.5 g; 3.65 mM) was dissolved in 10 ml of DMF. Consequently 1,11-dibromondecane; (0.52 g; 1.64 mM) was added. The reaction time was 21 hours at 100 ˚C. Cooled down to laboratory temperature, left in the fridge overnight, filtrated and purified in MeCN. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 588.38

Yield: 42 %.

M.p.: 256 – 258 °C

^{1}H NMR (300 MHz, DMSO d6) δ (ppm) 10.95 (s, 2H, =C=NOH), 9.10 (d, 4H, *J* = 5.69 Hz, H – 2,2′, 6,6′), 8.32 (d, 4H, *J* = 5.63 Hz, H – 3,3′, 5,5′), 6.45 (s, 4H, -NH_2), 4.57 (t, 2H, *J* = 7.18, 7.18 Hz, N-CH_2), 1.90 (m, 4H, N-CH_2-CH_2), 1.24 (m, 14H, N-CH_2-CH_2CH_2) ^{13}C NMR (75 MHz, DMSO d6) δ (ppm) 147.68, 146.95, 144.46, 122.69, 60.02, 30.46, 28.71, 28.67, 28.32, 25.31

1-FZ-14

1,1'-(dodecane-1,12-diyl)bis[4-(*N*'-hydroxycarbamimidoyl)pyridinium] bromide

N'-hydroxypyridine-4-carboximidamide (0.5 g; 3.65 mM) was dissolved in 10 ml of DMF. Afterwards 1,12-dibromododecane (0.53 g; 1.64 mM) was added. The reaction time was 22 hours at 100 ˚C. Cooled down to laboratory temperature, left in the fridge overnight, filtrated and purified in MeCN. Checked for impurities on TLC, structure and purity confirmed by NMR.

M.w.: 602.40

Yield: 34 %.

M.p.: 260 – 262 °C

^{1}H NMR (300 MHz, DMSO d6) δ (ppm) 10.95 (s, 2H, =C=NOH), 9.10 (d, 4H, *J* = 5.68 Hz, H – 2,2′, 6,6′), 8.32 (d, 4H, *J* = 5.61 Hz, H – 3,3′, 5,5′), 6.45 (s, 4H, -NH_2), 4.58 (t, 2H, *J* = 7.11 Hz, N-CH_2), 1.90 (m, 2H, N-CH_2-CH_2), 1.24 (m, 18H, N-CH_2-CH_2CH_2)
^{13}C NMR (75 MHz, DMSO d6) δ (ppm) 147.69, 146.96, 144.48, 122.70, 60.03, 30.46, 28.80, 28.71, 28.33, 25.31

4.2. DETERMINATION OF ACTIVITY PARAMETERS

4.2.1. **Method**

To determine the AChE reactivation/inhibition capability of synthesized substances a rapid photometric method, known as Ellman´s method, was used. This spectrometric method is extremely sensitive and is applicable to either small amounts of tissue or to low concentrations of enzyme.

In this method acetylthiocholine, analog of the naturally appearing enzyme, is used as the substrate.

The principle of this method is the rate measurement of production of thiocholine as acetylthiocholine is hydrolyzed by AChE *(I)*. This is accomplished by the continuous reaction of thiocholine with 5-dithiobis-2-nitrobenzoate ion to produce yellow anion of 5-thio-2-nitrobenzoic acid *(II)*. The rate of color production is spectrofotometrically measured at 412nm.(34)

4.2.2. **Measuring Protocol**

The reactivation/inhibition ability was measured on multichannel spectrophotometer Sunrise (Tecan, Salzburg, Austria). The standard polystyrene microplates with 96 wells (Nunc, Rockilde, Denmark) were chosen as reaction cuvettes. Human erythrocyte AChE (Sigma-Aldrich) was used throughout experiments. Pesticides paraoxon (POX) was purchased from (Sigma-Aldrich). 50 mM phosphate buffer pH 7.4 was used throughout all experiments.

The activity of the enzyme was adjusted up to 0.002 U/μl. One well was filled with the consequent chemicals – enzyme solution (15 μl), phosphate buffer (60 μl), 0.4 mg/ml 5,5´-dithiobis(2-nitrobenzoic)acid (DTBN; 20 μl,). The enzyme was inhibited *via* addition of

10^{-4} M POX, (5 μl) and 10^{-6} M VX in pure propan-2-ol or propan-2-ol itself as a control. The mixture was left for five minutes. After inhibition, cholinesterase was reactivated by addition of 10^{-4} M/10^{-5} M oxime-reactivator solved in the phosphate buffer. Enzyme activity was measured after 15 minute incubation *via* addition of 1 mM acetylthiocholine chloride (20 μl, ATChCl). Oximolysis was determined similarly by displacing enzyme with the phosphate buffer containing 1 mg/ml albumine. The microplate was gently shaken by the incorporated robotic system just before measurement. Absorbance was measured against phosphate buffer at 412 nm.

The reactivation ability was calculated according the subsequent equation.

$$(\%) = \frac{A_r - A_{ox}}{A_0 - A_i} \times 100$$

A_r indicates absorbance at 412 nm provided by cholinesterase reactivated by reactivator; A_{ox} – absorbance provided by oximolysis; A_0 – absorbance provided by intact cholinesterase; A_i – absorbance provided by inhibited cholinesterase.

All measurements were made in triplicate and the reactivation data were expressed as average value ± standard deviation (SD).

4.2.3. **Reactivation Results**

All substances were tested for reactivation in two concentrations (10^{-4} M, 10^{-6} M). VX and paraoxon were used as AChE inhibitors, for comparison purposes obidoxime was drafted. Analyzed substances with test results, which are given as an average value of three independent measurements, are listed in Tab.3

To determine the optimal concentrations of VX and paraoxon for measurements, calibration curve was essential. Concentration range of both entities was prepared and the amount inhibition for each concentration was determined, thus obtaining enough data for calibration curves. (Fig.1, Fig.2)

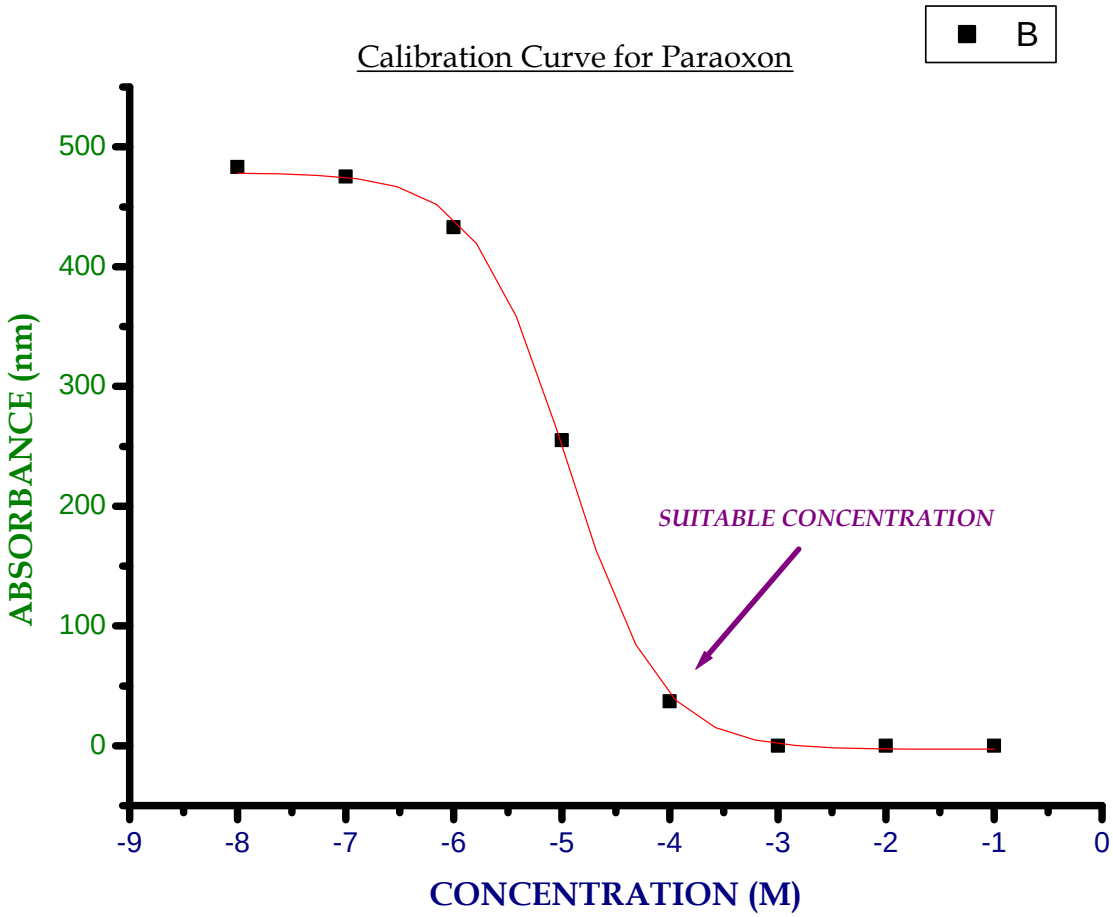


Fig.1 Calibration curve for paraoxon

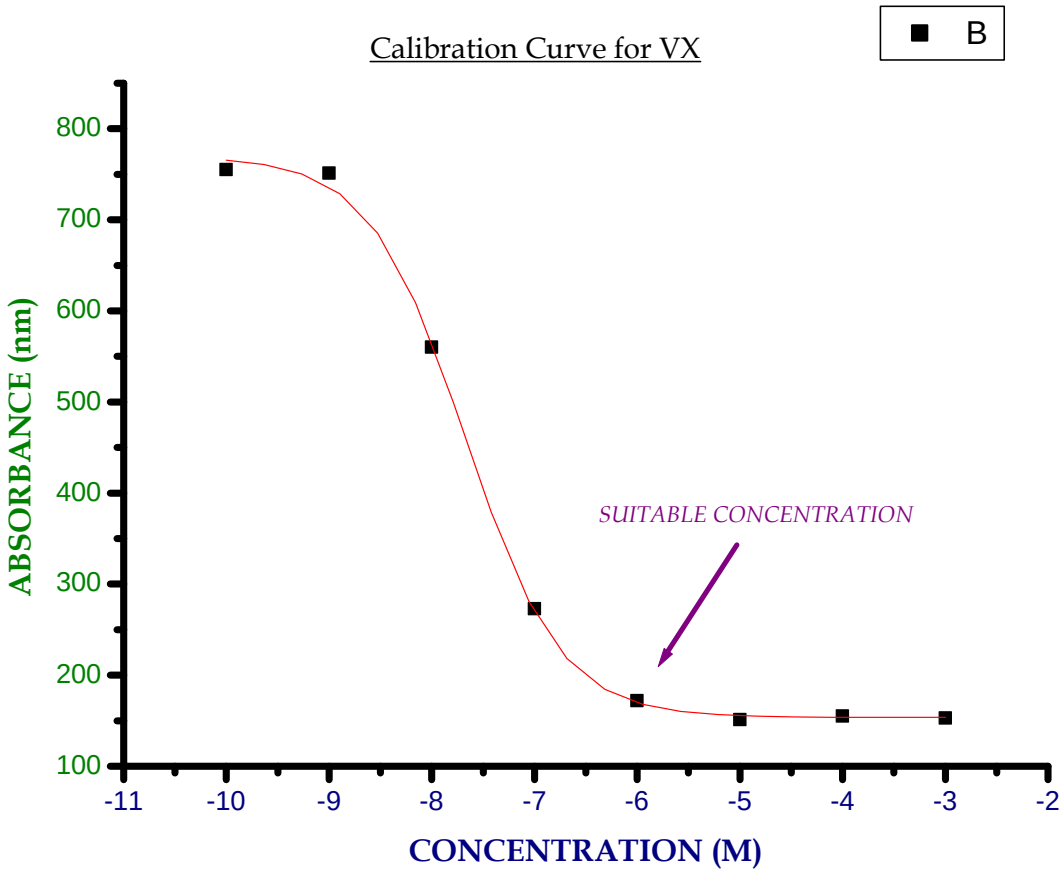


Fig.2 Calibration curve for VX

Tab. 3 **REACTIVATION OF AChE (%))**

	VX		PARAOXON	
	10^{-4} *M*	10^{-6} *M*	10^{-4} *M*	10^{-6} *M*
OBIDOXIME	40.00 ± 1	10.00 ± 0	70.00 ± 2	20.00 ± 3
1-FZ-1	-5.00 ± 0.3	**4.80 ± 1.1**	-5.00 ± 0.4	-3.0 ± 1.9
1-FZ-4	**1.30 ± 1.8**	-8.00 ± 1.6	**8.80 ± 1.5**	-1.00 ± 1.4
1-FZ-5	-10.00 ± 1.6	-5.00 ± 0.6	**8.20 ± 1.2**	**1.80 ± 1.7**
1-FZ-6	-4.00 ± 0.6	-3.00 ± 1.8	**4.09 ± 1.6**	**0.90 ± 0.6**
1-FZ-7	0.5 ± 1.2	-0.23 ± 1	4.50 ± 2.4	12.30 ± 1.8
1-FZ-8	**4.90 ± 5**	**4.10 ± 0.3**	-1.00 ± 1.5	**1.30 ± 1.8**
1-FZ-9	**0.70 ± 0.9**	0.00	-4.00 ± 1.6	-3.00 ± 0.3
1-FZ-10	**0.70 ± 1.6**	**1.2 ± 0.9**	-7.00 ± 1.3	**6.30 ± 1**
1-FZ-11	**3.50 ± 1.7**	**0.10 ±** 1.6	**1.30 ± 0.2**	**1.90 ± 0.7**
1-FZ-12	0.00	0.00	**0.20 ± 1.3**	**0.40 ± 0.5**
1-FZ-13	-3.00 ± 0.8	-4.00 ± 0.4	**0.10 ± 2.4**	**0.70 ± 2.7**
1-FZ-14	-3.00 ± 0.8	-2.00 ± 0.3	**4.30 ± 4**	**5.60 ± 1.2**
1-FZ-15	**1.40 ± 1.1**	0.00	**0.40 ± 1.1**	**1.30 ± 0.3**
1-FZ-16	0.00	-1.00 ± 0.4	**0.8 ± 0.4**	-1.00 ± 0.4
1-FZ-27	non soluble		non soluble	
1-FZ-28	**2.50 ± 0.6**	**0.50 ± 1**	**8.00 ± 0.8**	**6.20 ± 1.3**
1-FZ-29	-1.00 ± 1.6	-1.00 ± 0.4	**2.20 ± 1.9**	**0.20 ± 1.2**
1-FZ-30	-3.00 ± 1.6	**1.20 ± 1.1**	**0.50 ± 1.4**	**1.5 ± 0.3**
1–FZ– 34	**1.50 ± 1.2**	-2.60 ± 1.5	**8.00 ± 0.5**	**2.00 ± 0.5**
1– FZ- 35	0.00	**4.60 ± 1.6**	**10.40 ± 1.7**	**6.00 ± 1.3**

4.2.4. **Inhibition Results**

Ellman´s method was adapted for the measurement of inhibition. Each substance was prepared in concentration range starting from 10^{-2} M till 10^{-9} M. Except substance *1-FZ-27;* which could not be dissolved. Obtained absorbance was used to calculate IC_{50} in program ORIGIN from dose- response curve. Obtained results are shown in Tab.4.

In Fig.3 and 4 examples of dose-response curves used for calculations of IC_{50} are shown.

Tab. 4 **INHIBITION CONCENTRATIONS (IC_{50})**

INHIBITION of AChE (IC_{50}, M)	
1-FZ-1	$1.30 . 10^{-3}$
1-FZ-4	$1.33 . 10^{-3}$
1-FZ-5	$9.13 . 10^{-3}$
1-FZ-6	$1.21 . 10^{-3}$
1-FZ-7	$1.86 . 10^{-2}$
1-FZ-8	$1.24 . 10^{-4}$
1-FZ-9	$1.92 . 10^{-4}$
1-FZ-10	$3.27 . 10^{-5}$
1-FZ-11	$5.78 . 10^{-5}$
1-FZ-12	$7.85 . 10^{-6}$
1-FZ-13	$1.12 . 10^{-5}$
1-FZ-14	$4.31 . 10^{-5}$
1-FZ-15	0.72
1-FZ-16	104.81
1-FZ-27	not soluble
1-FZ-28	$2.80 . 10^{-4}$
1-FZ-29	0.72
1-FZ-30	$8.59 . 10^{-4}$
1-FZ-34	$5.23 . 10^{-3}$
1-FZ-35	$2.32 . 10^{-3}$

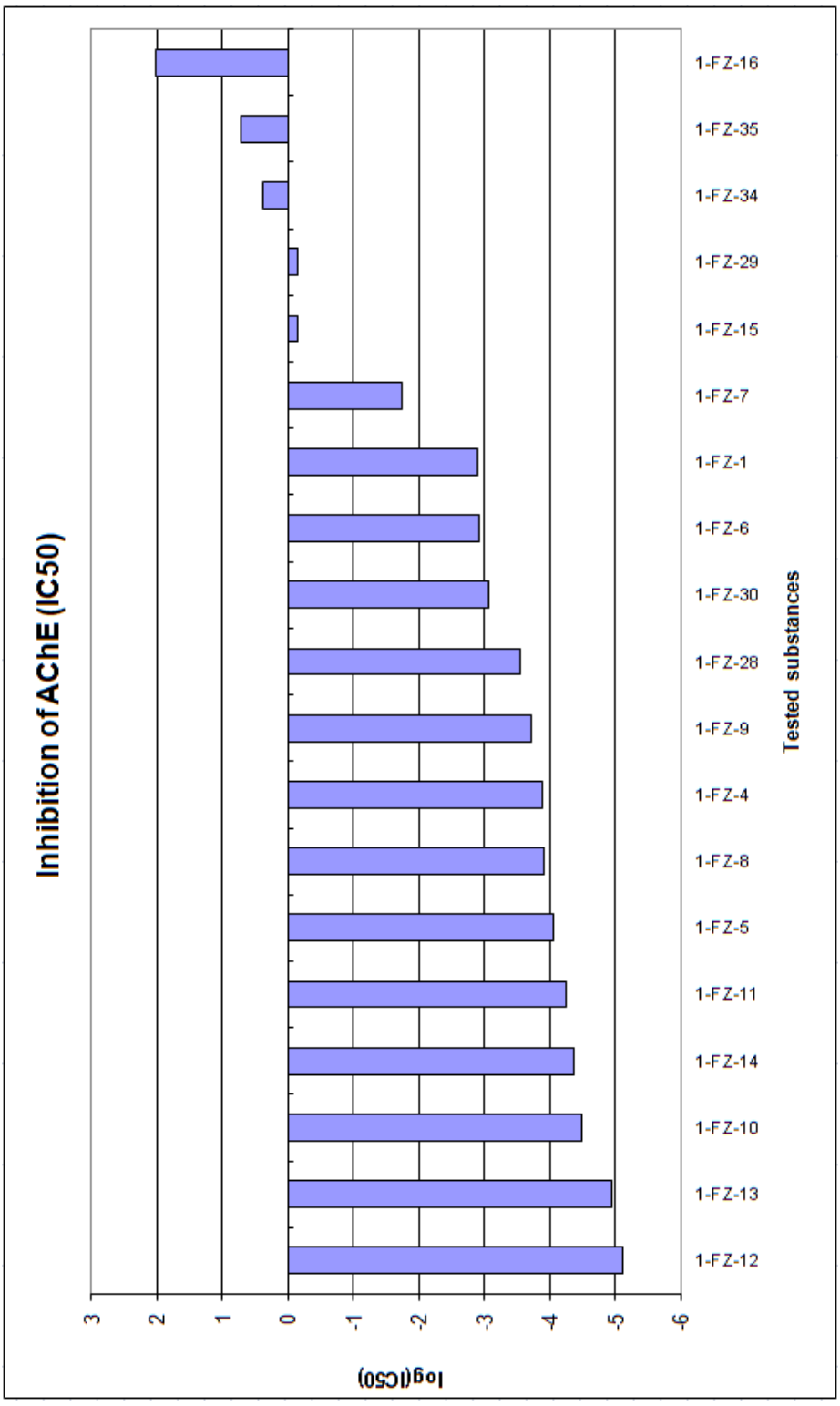


Fig. 3 Tested Substances vs. IC_{50}

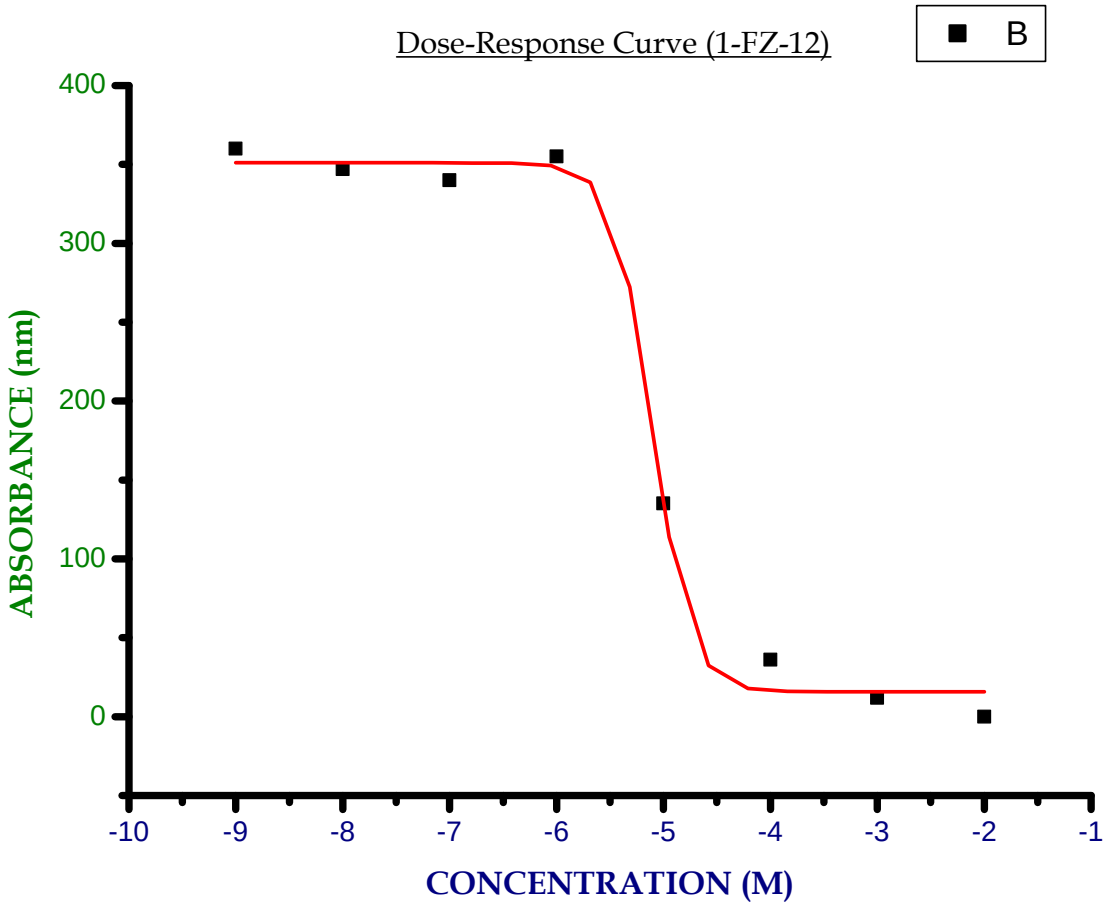


Fig.4 Example of Dose–Response Curve from ORIGIN.

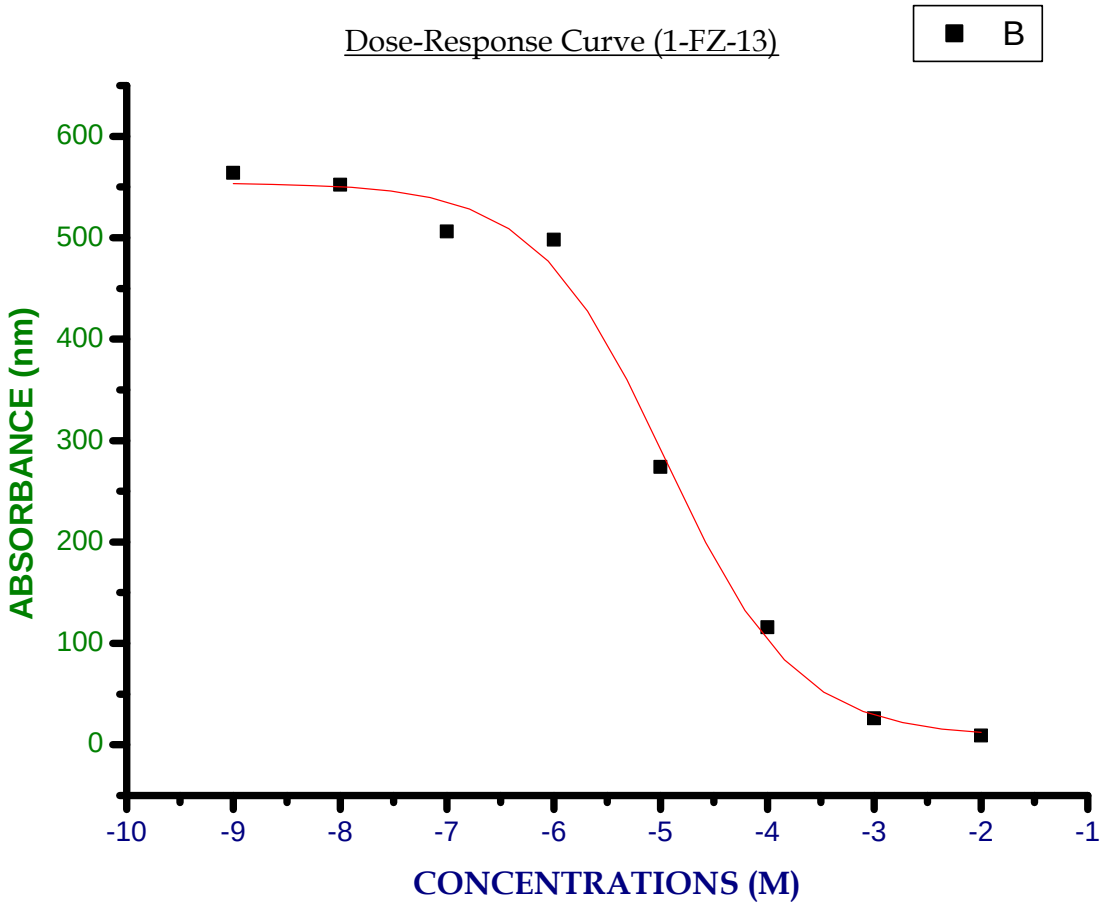


Fig.5 Example of Dose–Response Curve from ORIGIN.

5. DISCUSSION

Reactivation of AChE depends on many factors such as structure of OPC and of the potential reactivator, time between inhibition and application of the reactivator, lipophilicity, means of degradation in the body etc. New substances to be admitted for *in vivo* testing must show at least 10% reactivation activity in *in vitro* models.

Two different kinds of substances were synthesized: monoquaternary and bisquaternary compounds with an oxime group modified to amidoxime group. The compounds varied in the lengths of either side chain or linking chain and were tested for reactivation/inhibition properties.

Reactivation was tested on AChE inhibited with VX and paraoxon. From calibration curves the most suitable inhibition concentration was selected and used for testing (VX – 10^{-6} M, paraoxon – 10^{-4} M. For newly synthesized substances standard concentrations of 10^{-4} M and 10^{-6} M were used.

VX is a nerve agent which inhibition of AChE is quite easily reversed with most substances. Unfortunately none of the newly synthesized compounds proved to be a very potent reactivator. The highest reactivation (4.90%) showed 1-FZ-8; surprisingly even the milder concentration showed reactivation activity. Also compounds 1-FZ-1 (4.80%) and 1-FZ-35 (4.60%) showed some reactivation ability. Amazingly this activity was shown by the milder concentration; this can be due to the lower oximolysis of the oxime group with lower concentrations. Unexpectedly there is no correlation between the length of the side chain or linking chain and reactivation potency.

Paraoxon is a pesticide with not as strong inhibition potential as VX. This can be deduced from the reactivation results, as nearly all compounds showed some reactivation ability of AChE. The most potent proved to be 1-FZ-4 (8.80%), 1-FZ-5 (8.20%), 1-FZ-28 (8.00%) and 1-FZ-35 (10.40%). But when these results are compared to the reactivation potential of obidoxime, no farther research on these structures can be

expected. Once again we cannot derive any rules about dependence of activity on the structure.

None of these compounds had showed significant oximolysis, which can be due to the presence of NH_2 group on carbon atom in the oxime group. This modification can have protective ability, thus making it more resistant to oximolysis.

Since reactivation potential of these compounds has not been very significant, IC_{50} was measured to determine if their ability is not in the field of inhibition. From the results in Fig. 3., it can be seen very interesting inhibition activity for some of the compounds, especially for 1-FZ-12 and 1-FZ-13. From this graph we can derive an interesting dependence of IC_{50} on structure. The most potent inhibitors are the monoquaternary substances, whereas bisquaternary are mostly poor inhibitors.

6. CONCLUSION

All together 20 monoquaternary/bisquaternary new compounds were prepared, with different lengths of carbon side or linking chains.

Their reactivation and inhibition activity has been tested against VX and paraoxon. None of the substances proved to be a potent reactivators with possible clinical application. Some compounds displayed a very low reactivation % (1-FZ-4 , 1-FZ -8, 1-FZ-1, 1-FZ-28, 1-FZ-35) but nothing that would be interesting for father tests.

Quite a lot of the substances showed inhibition potential but most significant inhibition was displayed by monoquaternary substances: 1-FZ-12 and 1-FZ-13.

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8. SUMMARY

Originally organophosphorus compounds were used as pesticides. Unfortunately, their irreversible inhibition of AChE was discovered, and they were widely misused as warfare agents.

Presently, there is not a reactivator good enough to reverse completely irreversible inhibition of AChE. Thus, especially for military purposes, it is very important to look for better reactivators.

As the reactivators can both reactivate inhibited AChE and inhibit the intact AChE, they have also been tested as potential therapeutics for Alzheimer's disease.

New modification of oxime group with $-NH_2$ was tried for better reactivation or inhibition results compared to widely used conventional drugs. Also monoquartenary and bisquartenary modifications were tested for possible improvement in activity. Wide range of substances was synthesized, which differed in homologous unit $-CH_2-$. Thus substances with short aliphatic side chains, with partly hydrophilic properties, and those with long chains with mainly liphophilic properties as well as bisquarternary derivatives with various linking chains between two pyridininum centers were prepared and tested.

Unfortunately these modifications did not prove to have any positive effect on activity or on inhibition.

Some substances have shown mild reactivation possibility or inhibited AChE at low concentrations but if compared to therapeutically already use drugs, the activity is not significant.

9. SOUHRN

Původně měly být organofosforové látky používány jako pesticidy. Bohužel byly objeveny jejich inhibiční vlastnosti na AChE, což se začalo zneužívat pro výrobu bojových látek.

V současné době neexistuje ideální reaktivátor, jenž by kompletně obnovil aktivitu AChE po ireverzibilní inhibici. Proto je speciálně pro vojenské účely důležité hledat novější a zároveň účinnější látky.

Protože reaktivátory mohou jak reaktivovat inhibovanou AChE, tak inhibovat intaktní AChE, jsouv posledních letech testovány jako potenciální terapeutika Alzheimerovy choroby.

Nová modifikace oximové skupiny pomocí $-NH_2$ byla zkoušena za účelem hledání nových látek s lepší reaktivační nebo inhibiční aktivitou, než mají v současnosti používané sloučeniny. Zároveň se zkoušelo, jestli aktivitu ovlivní, bude-li sloučenina monokvarterní nebo biskvarterní. Bylo připraveno široké spektrum látek, jenž se liší od sebe homologní jednotkou $-CH_2-$.

Sloučeniny tímto způsobem vzniklé mají buď krátké postranní řetězci a slabě hydrofilní povahu nebo dlouhé postranní řetězce a silně lipofilní charakter. Byly syntetizovány i biskvartétní sloučeniny s různými řetězci spojujícími dvě pyridiniová centra.

Bohužel žádná z modifikací neprokázala pozitivní vliv na účinek ani na inhibici AChE.

Některé látky prokázaly mírný účinek, ale v porovnání s již používanými látkami nebyl výrazný.