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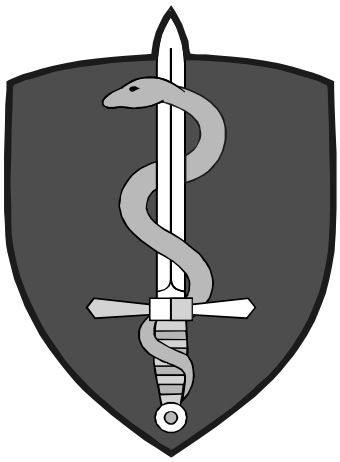

Interaction of organophosphorus poisoning antidotes with muscarinic and nicotinic receptors

Dissertation thesis

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Abbreviations:

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChEi	Acetylcholinesterase inhibitors
BBB	Blood brain barrier
BPM	Beats per minute
CNS	Central nervous system
GPCR	G-protein coupled receptor
HACU	High Affinity Choline Uptake
mAChRs	Muscarinic acetylcholine receptors
NMS	N-methylskopolamine
nAChRs	Nicotinic acetylcholine receptors
OP	Organophosphorus/organophospahte
OPi	Organophosphorus inhibitors
QNB	Quinuclidinyl benzilate

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1 Problem and its solving

Organophosphorus inhibitors (OFi) of acetylcholinesterase (AChE) represent an extremely toxic group. Members of this group can be used as a military weapon or misused for terroristic purposes. We can also find their utilization in agriculture as pesticides and in the industry (plasticizers etc.). These compounds inhibit AChE at the serine hydroxyl group at its active site. The inhibited enzyme cannot fulfill its physiological role in the organism – i.e. splitting the neuromediator acetylcholine ACh at the synaptic clefts. Consequently, ACh accumulates in the cholinergic synaptic junctions. The poisoning manifests as a cholinergic syndrome. In cases of insufficient treatment, death is caused by paralysis of the respiratory muscles and of the respiratory center in the brain [1, 2].

Symptomatical treatment of the OF poisoning is provided by two main groups of drugs. (1) Anticholinergics, like atropine, are able to antagonize the effects of excessive ACh by a blockade of muscarinic receptors. (2) Reactivators of AChE - oximes - are able to restore the physiological function of inhibited AChE. In addition, diazepam or other benzodiazepines can be used as anticonvulsants. Anticholinergics and reactivators are usually administered together because of their synergistic effect [3, 4]. Today, HI-6 and obidoxime are the most commonly used oxime reactivators in the treatment of organophosphorus poisoning. However, the reactivators differ in their efficacy against individual nerve agents and no universal antidote has been developed yet. Moreover, the problem of "aging" is also an important issue. Owing to this fact, new, versatile AChE reactivators, are thus required, or alternative treatment approaches may be introduced.

Such an alternative approach presumes other oximes´ mechanism of action, not related to the reactivation [5]. Oximes have been reported to act at several levels of the cholinergic transmission including synthesis, release, inactivation and re-uptake of the transmitter, but the interaction with muscarinic and nicotinic receptors, has been put forward as the most plausible alternative of mechanism of action.

The aim of this dissertation project deals with the interaction of oxime reactivators with muscarinic and nicotinic receptors. The experimental part has been based on the review of available literature [6] and include both *in-vitro* and *in-vivo* testing in order to investigate their antimuscarinic and antinicotinic properties. The differences in efficacy and anticholinergic potency of the individual reactivators, and further, the correlation between *in-vitro* and *in-vivo* effects were examined. The review article and the obtained results, which show reactivators´ composite mechanisms of action has been published in scientific journals and presented in the form of lectures and posters at scientific conferences.

2 Review of literature

2.1 Cholinergic system

2.1.1 Cholinesterases

Cholinesterases belong to a family of hydrolases and are able to split the ACh in the synaptic cleft to choline and acetic acid (Fig.1). These products are reused for the synthesis of new ACh. Two structurally and functionally very similar enzymes occur in a body. Acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BuChE; EC 3.1.1.8). Both catalyze hydrolysis of ACh with similarly high efficiency but they differ in the distribution in the body and in the affinity to larger substrates. BuChE occur in the blood plasma, kidney and liver and is able to more effectively hydrolyze esters with larger moieties. Its role in the organism is still unclear but it is suggested that it degrades some esters, which arise from metabolism. Interestingly, mice lacking the AChE gene but having normal BuChE activity can survive more than one year, but are more sensitive to organophosphate (OP) poisoning. This suggests that BuChE may substitute at least in part for the lack of AChE activity [7]. Generally, BuChE also binds bulky ligands. AChE, that plays a key role in cholinergic neurotransmission, is mainly found in the central nervous systems, neuromuscular junctions, and the hematopoietic system of vertebrates [8]. Cholinesterase activity has been also observed in some plants [9], seaweed [10], fungi [11] and in virus [12] .

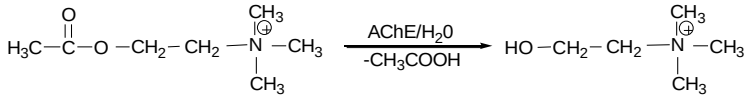


Figure 1: Cleavage of ACh to choline and acetic acid

Both enzymes in vertebrates are encoded by one gene. Even though the primary structure differs interspecially, the structure of active sites essential for catalysis of ACh differs very little. Catalytic subunits of both cholinesterases consist of a single 500 to 600-amino acid-long peptide. The site of catalysis is buried 20 Å deep into the center of the globular catalytic subunit [8]. The key unit at the active site of the human enzyme, which is responsible for the enzymatic reaction, is the catalytic triad and consists of 3 aminoacids (Ser200, His440, Glu327) [13]. The oxyanion hole (Gly18, Gly119, Ala201), that is localised in the vicinity of the triad, stabilizes carbonyl oxygen of ACh during hydrolysis [14]. Binding sites are the acyl pocket, the choline binding site, and the peripheral site. The acyl pocket and the choline binding site are located next to the active serine, controlling the size of ligands. The peripheral site (Tyr70, Tyr121, Trp279) is located at the rim of the gorge, approximately 14 Å from the active serine. It binds cationic and aromatic inhibitors that are too large to enter the gorge, such as gallamine or bisquaternary ligands (reactivators).

2.1.2 Cholinergic receptors

There are two types of receptors for ACh, muscarinic and nicotinic. While nicotinic receptors occur at the neuromuscular end-plate and in the autonomic ganglion, muscarinic receptors occur on smooth muscle and glandular cells as well as on nerve terminals [15, 16].

2.1.2.1 Muscarinic receptors

Five known subtypes (M_1-M_5) of the muscarinic acetylcholine receptors (mAChRs) have been distinguished. They belong to the G-protein coupled receptor (GPCR) superfamily. The M_1, M_3 and M_5 subtypes are coupled to the activation of phospolipase C through G-protein leading to the activation of inositolphosphate cascade of second messengers. They are extensively expressed on postsynaptic terminals where they facilitate fast synaptic transmission and metabotropic functions [17]. The muscarinic M_2 and M_4 subtypes may be regarded as inhibitory since they may reduce cAMP formation and prolong potassium channel opening. They also act as inhibitory autoreceptors on presynaptic terminals, thus inhibiting the neurotransmitter release from presynaptic terminal. On the other hand, M_1 subtype localized presynaptically may enhance ACh release from the nerves [18]. M_1 mAChRs are widely expressed in the brain, but significant levels have also been reported in salivary glands and sympathetic ganglia [19, 20]. M_3 mAChRs are found extensively in smooth muscles of the GIT and urinary tract, where they play a major role in contraction and in secretion of exocrine glands [16]. M_5 mAChRs has been identified in recent years in various peripheral and cerebral blood vessels [20, 21]. M_2 mAChRs are abundantly expressed in the heart, smooth muscle organs and in the caudal formation of the brain. M_4 mAChRs are widely expressed in lungs, brain and in lower concentration in the salivary glands and ileum. The study of mAChRs is complicated due to the low degree of selectivity of agonists and antagonists. Absolutely selective compound for individual subtype does not exist [22].

2.1.2.2 Nicotinic receptors

Nicotinic receptors belong to the ion-channel coupled receptor family and are responsible for fast excitatory synaptic transmission at autonomic ganglia, neuromuscular junctions and at various locations in the CNS. Two major classes of nicotinic acetylcholine receptors (nAChRs) have been distinguished. Neuronal-type and muscle-type. The classes differ in molecular structure and pharmacological properties. Furthermore, each class can be divided into subclasses, that also exert pharmacological heterogeneity too e.g. different ligands exist. The neuronal subtype forms a heterogeneous family of ion channels, which are differently expressed in many regions of the CNS and peripheral nervous system. These different receptor subtypes, which have characteristic pharmacological and biophysical properties, have a pentameric structure consisting of the homomeric or heteromeric combination of 12 different subunits (α2–α10, β2–β4) [23]. The muscle-type receptors

mediate ion permeation at the neuro-muscular endplate. They are pentameric complexes, consisting of four distinct protein subunits (α1β1γδ) in the ratio of 2α:1β:1γ:1δ [24]. An epsilon subunit is substituted for the gamma subunit during development, altering the functional response of the receptor [25]. It has been also reported that nAChRs are present in a number of non-neuronal cells, where they play a significant functional role [23].

2.2 Organophoshorus poisoning

2.2.1 Inhibitors of AChE

There are two main groups of inhibitors – reversible and irreversible. Reversible inhibitors rapidly form non-covalent complexes with AChE at the active center or at the peripheral site or they may bind to both of them at the same time. These inhibitors can be drugs (e.g. donepezil, tacrine, physostigmine), oxime reactivators (HI-6, obidoxime) or ACh itself. On the other hand, irreversible inhibitors form a covalent chemical bond with the hydroxyl group of serine at the active site of the enzyme. Organophosphates (OP) and carbamates are common irreversible inhibitors of AChE [8].

2.2.1.1 Organophosphates

OPs represent an extremely toxic group of compounds. Members of this group can be both nerve agents (e.g., sarin, tabun, soman, cyclosarin, and VX) (Fig. 2) and pesticides (e.g. parathion, paraoxon, chlorpyrifos, malathion). These compounds inhibit AChE via its phosphorylation or phosphonylation at the serine hydroxyl group at its active site. The inhibition of the enzyme then manifests as a cholinergic syndrome. Clinical effects of excessive ACh stimulation depend on their localization and the type of receptor that is affected. Peripheral and central nervous symptoms exerted by muscarinic and nicotinic receptors are characteristic for organophosphate poisoning [1]. When an individual is exposed to a nerve agent, dose-dependent symptoms can be observed. These symptoms vary according to which receptor was affected. Typical muscarinic symptoms are dyspnoe, nausea, vomitus, diarrhea, miosis, hyperactivity of urinary bladder and hypersecrection of glands. Nicotinic symptoms manifests as weakness, tremor, tetanus and paralysis of striated muscles. Headache, slurred speech, hallucinations, anxiety and fuzziness are results of central poisoning. Paralysis of respiratory muscles and respiratory center in the brain is considered to be the major toxic effect that may lead to death [4].

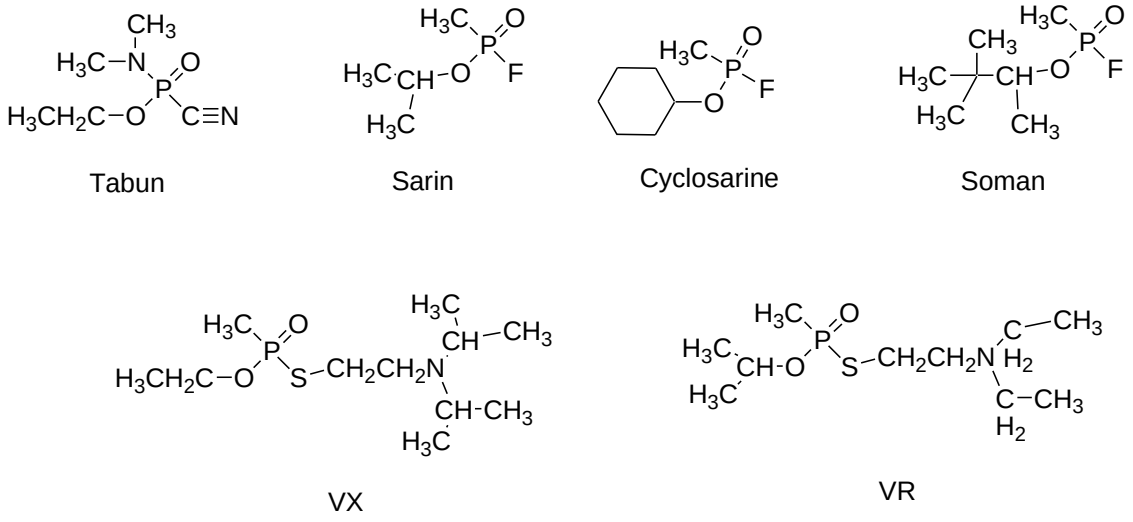


Figure 2: Structures of OP nerve agents

The OP inhibition of AChE, and in particular the efficacy of its treatment, is complicated by the so called "aging" (Fig. 3). Aging is a loss of an additional alkyl substituent group from the enzyme-inhibitor complex [26]. This dealkylation depends on the length of contact with inhibitor and on the structure of inhibitor [1]. Primary alkyls ages slowly, while secondary, especially branched alkyls undergo aging rapidly (e.g., soman). In other words, aging is a period of time within it is possible to reactivate the inhibited AChE. Aging of nerve agents such as soman is very fast and occurs in a few minutes, whereas aging of pesticides such as paraoxon or ethyl-dichlorvos can take days [27].

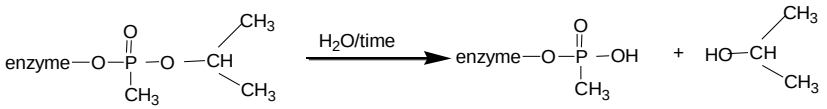


Figure 3: Aging of the inhibited enzyme

2.2.2 Therapy of OP poisoning

The strategy for therapy of poisoning has to be considered out of its relation to the time point of exposure. At an early stage, before exposure, the strategy could be considered as prophylaxis, while at a later stage, it needs to have treatment approach.

2.2.2.1 Prophylaxis

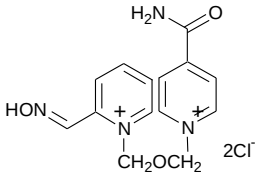
Prophylaxis is an option when an exposure to OP is expected. Various approaches are available [3]. Carbamates (pyridostigmine, physostigmine, aminostigmine) are reversible inhibitors of AChE with higher affinity than Ops, i.e. AChE can not be inhibited irreversibly. After spontaneous recovery normal AChE serves as the enzyme source. Carbamates can be administered together with cholinolytics, as benactyzine and trihexyphenidyle (PANPAL),

which reduce adverse effect of pyridostigmine on the periphery and reduce toxic effect of OP in CNS. Enzymes capable to split OP compound are another possibility. Such an enzyme is paraoxonase [28], which is still under development. Thirdly, scavangers are able to bind molecules of OP and by that protect AChE from the binding of the OPs. These compounds are exogenously administered. Artificially developed AChE and BuChE seems to be very effective as the prophylactic agents in this way [3]. Furthermore, the current standard oxime reactivators currently used in the post-exposure treatment exert prophylactic effects too. However, other routes than standard administration is necessary to ensure a prolonged duration of the effect. Transdermal administration of one of the most effective reactivator (HI-6) has been introduced into the Czech Army as TRANSANT [3].

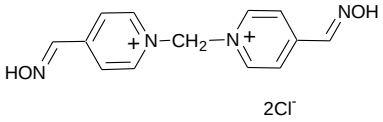
2.2.2.2 Treatment

The treatment is ensured by two functionally different groups of drugs. (1) Reactivators of AChE - oximes - are able to restore the physiological function of inhibited AChE. (2) Anticholinergics, like atropine, are able to antagonize the effects of excessive ACh by a blockade of muscarinic receptors. Anticholinergics and reactivators can be administered together because of their synergistic effect. In addition, diazepam or other benzodiazepines can be used as anticonvulsants [4].

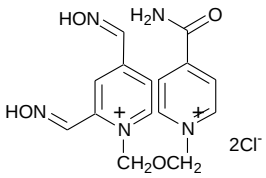
Atropine is still a major anticholinergic agent. However, it blocks muscarinic receptors mainly in the periphery, and it lacks nicotinic antagonism [29]. The advantage of atropine is a high tolerance at high, life-saving doses (more than 100 mg during 24 hours). For better BBB penetration biperiden, benactyzine and scopolamine [30] may be applied.



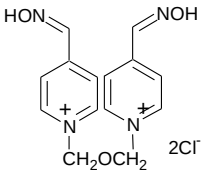
HI-6



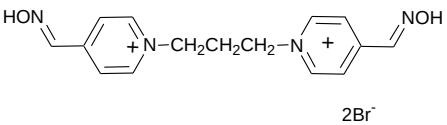
Methoxime



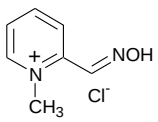
Hlö-7



Obidoxime



Trimedoxime



Pralidoxime

Figure 4: Commonly used oxime reactivators

Reactivators of AChE (Fig. 4) contain oxime moiety attached to a quaternary nitrogen pyridinium ring and are by this able to reactivate AChE inhibited by OP agents. The quaternary nitrogen is attracted by the anionic site of AChE and the reactivation occurs through nucleophilic interaction of the phosphorous by the oxime group (Fig. 5).

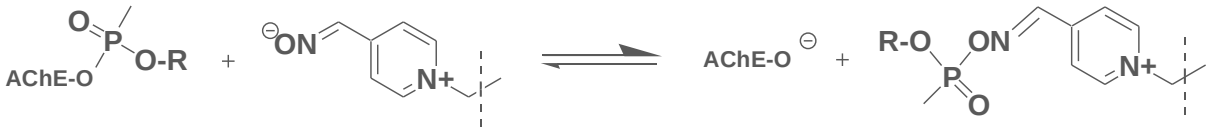
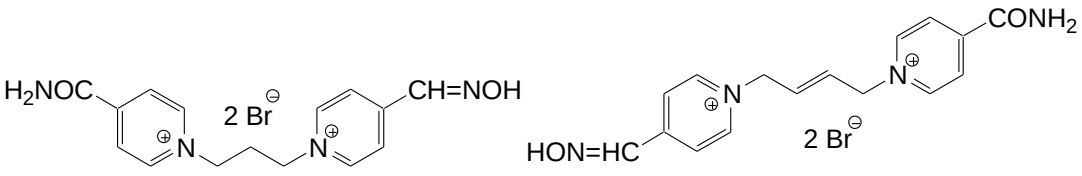


Figure 5: Reactivation of inhibited AChE by oxime. AChE inhibited by organophosphorus compound being nucleophilicly attacked by an oxime moiety of pyridinium reactivator. AChE structure is restored and the reactivator binds the organophosphate.

The antidotal treatment by oximes is still limited due to the following reasons: 1. no versatile antidote capable to effectively restore activity of AChE inhibited by random organophosphates has been developed yet. 2. Oxime reactivators are efficient only when administered before the "aging". HI-6 and obidoxime are the most commonly used oxime reactivators in the treatment of organophosphorus poisoning. Moreover, HI-6 is considered as the most effective and is a broad-spectrum antidote against soman, sarin and VX [4], but it is less effective against tabun [31]. On the other hand, obidoxime is used against poisoning by tabun as well as by pesticides. Trimedoxime can be used for tabun poisoning as well. Pralidoxime can handle poisoning by sarin and VX, but in other cases is not as effective as H oximes (HI-6, Hlö-7) [4]. Some sources consider pralidoxime as obsolete or at least controversial [32, 33]. Methoxime, which is commonly used in the USA, exhibits lower reactivation potency against soman and tabun, but exerts good efficacy against sarin and VX [4].

The high therapeutic potency of HI-6, besides its reactivating potency, might also be due to other antidotal mechanisms such as direct antimuscarinic and antinicotinic actions, restoration of neuromuscular transmission, retardation of the formation of the aged inhibitor-enzyme complex and inhibition of ACh release [34, 35].

In addition to these "standard reactivators" some new promising compounds have been developed such as K027 and K203 (Fig. 6). K027 is a very effective reactivator of methylparaoxone-inhibited AChE [36-38]. Moreover, since it has a lower toxicity than obidoxime, it may lead to a replacement of obidoxime as the preferred antidote in the treatment of poisoning by pesticides. K203 is also an effective compound in cases of tabun-inhibited AChE. The results from *in-vitro* as well as *in-vivo* examinations in rats indicate that K203 might be utilized in the treatment of tabun-poisoning [39].



K027 K203

Figure 6: Structures of newly developed recativators.

2.3 Interaction of oximes with muscarinic and nicotinic receptors

It is evident, that the major mechanism of action of the antidotes is the reactivation of inhibited AChE. However, other mechanisms may be involved. The data derived from tabun or soman lethally intoxicated primates suggest that, due to the lack or low reactivation potency of some agents, other mechanisms, not related to reactivation, may lead to survival via recovery of neuronal transmission in the respiratory centers and in the diaphragm [40, 41]. Similar life-saving results were obtained in a rat model, where rapidly aging crotylsarin was used and administration of HI-6 caused the recovery of neuronal transmission [42].

The exact pharmacological effects of oximes are still an open question. Oximes probably play a role at various levels of the cholinergic transmission, from the synthesis and release of Ach trough the interaction with pre- and post-synaptic receptors to the re-uptake of transmitters. Clement reported that oxime HS-6 inhibits the synthesis of ACh, the release of ACh and the re-uptake of choline in the chicken [43]. The release of ACh from presynaptic fiber can be modulated in both ways suggesting an interaction with presynaptic muscarinic [44] and/or nicotinic receptors [45] or via the interaction with the HACU transporter system (High Affinity Choline Uptake), which is a key-regulatory system in the synthesis of ACh [46]. In the latter case, the interaction with presynaptic muscarinic receptors is also suggested [47]. While a decrease in release have been reported in the rat brain [48], an increase has been reported in the rat striated muscle [49].

The interaction of reactivators with postsynaptic receptors has been described by many authors. In the treatment, some oximes have been reported in primates to be effective against soman poisoning even after aging [41] and some compounds are effective even when the compounds lack the oxime moiety [50, 51].

The effect on nicotinic receptors at the neuromuscular junctions was examined via the phrenic nerve-diaphragm model. HI-6 has been reported to counteract the failure of neuromuscular transmission caused by crotylsarin. Since HI-6 had similar effect as pure cholinergic receptor antagonists (gallamine, mecamylamine), which are not involved in AChE reactivation, nicotinic antagonism of this compound has been suggested. Furthermore, HI-6 has been shown to be able to counteract crotylsarin-induced excessive release of ^{3}H-ACh from an isolated endplate [5]. Tattersall examined the action of oximes on the nicotinic ion channel in the mice muscle endplate by using of single-channel recording. He suggested that pharmacological action against soman blockade *in-vitro* is due to the channel blocking activity of HI-6, obidoxime, trimedoxime and similar compounds [52]. Ganglion blocking properties has been suggested also by Lundy et al., since some oximes may cause a reduction of the blood pressure in cats [53]. Nicotinic receptors (and muscarinic as well) in the rat brain have been said to be blocked by some bispyridinium compounds as judged by displacement examinations with tritiated QNB [54]. However, single channel studies have revealed that HI-6 and pralidoxime increases the opening probability of nicotinic receptors that are activated by ACh [55].

Concerning the blocking properties of the muscarinic receptors, binding studies of some H-oximes has been performed on mice smooth muscle tissue. Their inhibitory constants

are in the micromole range. However, as observed also at nicotinic receptors, it does not correlate with the efficacy against soman *in-vivo* [56]. Functional muscarinic antagonism has been observed at guinea pig as well [57]. The mechanism of binding to the muscarinic receptors is still not fully understood, but an allosteric manner has been suggested [58, 59]. Obidoxime has been reported to be a competitive ligand at the common allosteric site [60, 61]. Moreover, *in-vitro* testing proved the oxime HGG-12 (and the analog HGG-42) to also be allosteric inhibitors [44]. Ellis and Seidenberg investigated the effect of obidoxime and other ligands (tacrine, gallamine) on rat cardiac muscarinic receptors and concluded that obidoxime is a weak allosteric inhibitor [60]. Cholinergic activity of oxime HGG-12 has been described as competitive antagonism without intrinsic activity at porcine atrial muscarinic ACh receptors. The stimulatory action on muscarinic receptors, which has been described by several authors, is therefore suggested to be due to partial inhibition of AChE by the oxime rather than to direct agonism at muscarinic ACh receptors [62].

Anti-AChE activity of bispyridinium compounds has also been observed when measured physiologically in the guinea pig ileum [63] or spectrofotometrically *in-vitro* [64] and therefore indirect stimulatory effects can be concluded to occur at a cholinergic system as well.

Thus, in can be concluded, that pharmacological effects of oximes are complex and not yet well defined. There is a hypothesis that oxime reactivators used in the treatment of OP poisoning exert both antimuscarinic and anticotinic properties. However some questions still need to be answered.

1. Are the non-esterase inhibitory properties of oximes significant for the treatment of OP poisoning and by which mechanisms do they exert the effects?
2. Are there differences among reactivators in anticholinergic efficacy?
3. Why is there a difference between *in-vitro* and *in-vivo* effects of the oximes?

This thesis tries to answer these questions. Therefore, two commonly used reactivators (HI-6, Obidoxime) and two newly synthesized promising compounds (K027 and K203) have been chosen in order to investigate their properties at different levels in the cholinergic transmission by employing in-vitro and in-vivo techniques.

3 Methodology

It is obvious that different approaches of investigation require different techniques. Methods that were used are described in detail in individual publications (appendix 10.1-10.8). Here is just a short summary, presented in a muscarinic and a nicotinic section, in order to explain the principle of applied procedures.

3.1 Muscarinic receptors

3.1.1 Binding study (10.7)

The affinity of reactivators has been measured. Radiolabeled ligand of muscarinic receptors N-methylscopolamine (NMS) was displaced by the tested compound. Higher affinity of tested compound results in larger decrease of radioactivity, which was measured in a scintillation counter. As source of muscarinic receptors, rat brain homogenates were used.

3.1.2 GPCR activation assay (10.7)

In order to get functional correlates to the binding examinations, the activation of G-protein coupled receptor (M2) was measured. A commercial kit based on measuring β-arrestin recruitment was used to reveal the transduction of the signal through the muscarinic receptors into the second messengers. Affinity of the tested ligands and its intrinsic activity were evaluated. Currently, the decrease in activation caused by oxotremorine-M (muscarinic agonist) was studied for various oximes (muscarinic antagonists).

3.1.3 Functional study *in-vitro*

Further functional correlates were searched for in *in-vitro* studies using tissue bath where isolated organs were placed after dissection from rats. The models enable studies in a more complex model. The tissue bath simulates a physiological environment where the whole organ can undergo examinations. Rat urinary bladders (M3 receptor occurrence, 10.1, 10.6) and atria (M2, 10.6) were used. By employing the muscarinic agonist methacholine, the receptors were stimulated to provoke smooth muscle contraction (bladder) or decrease in the heart frequency (atria). The responses were recorded in the presence and in the absence of tested reactivators. Interaction of reactivator and the AChE sometimes occurred, but since metacholine, which is poorly broken down by AChE, was used, this effect was expected to be small.

3.1.4 Functional study *in-vivo* (10.6)

In order to get indications for the clinical significance of different mechanisms, *in-vivo* studies were performed. The *in-vivo* examinations were performed on anesthetized rats. All drugs were administered via v. femoralis and the blood pressure and BPM were monitored via catheter introduced into the a. femoralis. The vagal nerve was stimulated via platinum electrode, which resulted in a drop of the heart frequency, due to the release of ACh from the nerve terminal and its effect on atrial muscarinic M2 receptors. The heart frequencies recorded in the presence and in the absence of tested reactivators and their effect were observed. The interaction with AChE may occur since endogenous ACh is broken down by AChE, which can be inhibited by reactivator.

3.2 Nicotinic receptors (10.8)

3.2.1 Patch clamp (*in-vitro*)

Nicotinic receptors are members of ion-channel family of receptors. The activated receptor is characterized by the current of ions (Na^+) through it. This current manifests as a change in amperage and the change can be measured via patch clamp technique. This method requires some carrier of the nicotinic receptor – currently TE671 cell line was used, which expresses a human embryonic muscle nAChRs. First, cells were stimulated by ACh to obtain a basal activation. Then ACh was administered together with a reactivator and the currents were compared.

3.2.2 Functional study *in-vivo*

The *in-vivo* functional studies were performed by measuring effects on nAChRs localized in the neuro-muscular endplate. By electrical stimulation of the innervation, muscle contractions were induced. The examination was performed on anesthetized rats. All the drugs were administered via v. femoralis and the muscle twitches were recorded via a thread fasten to the m. tibialis and to a recording device. The twitches were evoked by a platinum electrode placed to the nervus sciaticus. The twitches of m. tibialis ant. were recorded in the absence and in the presence of tested oxime in the whole-body circulation.

3.3 Additional studies

3.3.1 Interaction of oximes with AChE (10.1, 10.2, 10.3, 10.4, 10.6)

Oxime reactivators are potential AChE inhibitors, and therefore the effect of the oximes on the AChE needs to be quantified when the direct interactions at the receptor level are to be evaluated. The AChE inhibition may influence the receptor interaction indirectly. Often the inhibition of receptors and the inhibition of AChE are two contradictory processes. In order to assess the AChE interaction, the classical spectrophotometrical Ellman´s method based on the colored reaction of AChE with acetylthiocholine (substrate) and chromogen was applied.

3.3.2 Interaction of oximes with HACU (10.4)

The investigation of oximes and HACU has been published on just one reactivator (K112) in order to describe their complex mechanism of action. HACU system is a key regulatory step in the synthesis of ACh and is responsible for the transport of choline to the presynaptic cleft, where choline is used for new syntesis of ACh. In other words, inhibition of HACU leads to shortage of ACh in the synapsis, which may also be important in OP poisoning. Moreover, cooperation of M2 muscarinic autoreceptors with HACU is suggested.

4 Comment to the results

The aim of the thesis is to describe the interaction of oxime reactivators on the muscarinic and nicotinic receptors. First of all, the principle statement should be that, there exists an interaction before studying any mechanism in detail. This first step is fairly easily performed. However, to describe any interaction in detail is a little bit more complicated, since oxime reactivators exert very complexed mechanisms of action and have influence on various levels of cholinergic system. According to the literature and according to our observations, it may be concluded that oxime reactivators have antagonistic effect on both muscarinic and nicotinic receptors. However, cholinomimetic actions of these drugs can not be definitely excluded. There exist some evidences for the latter statement, which, however, depend on the method applied. In order to elucidate the mechanism of action, two standard reactivators (HI-6, Obidoxime) and two-newly synthesized drugs (K027, K203) were tested.

The binding study revealed the affinity of oximes to the the mAChRs. The affinity increases in following order HI-6<K203≈K027<Obidoxime. However, even the one with the highest affinity, obidoxime, could not reach the affinity of atropine which was 3-fold higher (10.7). This finding is in agreement with most other similar studies [56].

The binding to the mAChRs receptors is a prerequisite for the transduction of the signal trough the receptor, which, in the case of G-protein coupled receptors leads to the activation of the G-protein. In the GPCR activation based study (10.7), the oximes blocked the activation induced by oxotremorine (muscarinic agonist; control). No intrinsic activity of the oximes was observed and the blocking properties of the signal transduction increased in the following order: HI-6<K027<K203 ≈Obidoxime. This roughly corresponds to the binding affinities at least in that HI-6 has the lowest and obidoxime has the largest effect (10.7).

The *in-vitro* contraction study on the isolated rat urinary bladder showed antagonistic effect on M3 mAChRs (10.1, 10.4. 10.6). However, this effect was only obvious at higher concentrations of oximes. At lower concentrations, an opposite effect was observed (increase in contraction). This is probably due to an inhibition of AChE caused by the reactivators. In the atrial preparation, only the antagonistic effect on M2 mAChRs was observed (increase in heart frequency) (10.6., 10.7). An explanation to this fact seems not far to seek. For stimulation of M2 mAChRs methacholine (agonist), which is not broken down by AChE, was used i.e. potential inhibition of AChE did not result in the accumulation of agonist in the synapsis. However, methacholine was used to stimulate the bladders as well and here the effect on AChE was observed. This could be due to the fact that in the bladder, there is a pronounced non-neuronal production and a release of acetylcholine from the urothelium occurs. The mechanisms that induce the release is largely unknown, but is most likely to be induced by shear stress, i.e. stretch of the urothelium may be caused by the methacholine-evoked contraction [65]. Also, the activation of urothelial muscarinic receptors may contribute to the release. It means that the observed effect is due to the AChE inhibition and its influence on ACh´s break down, not that one of methacholine. Interestingly, the comparison of individual agents´ efficacy showed a high potency of HI-6 (and possibly obidoxime), which does not correlate with the affinity studies, where HI-6 showed the lowest

affinity to mAChRs. An explanation could be found in the interaction with presynaptic receptors, the HACU system or in another mechanism that influence the release of endogenous ACh.

The *in-vivo* muscarinic studies of the heart frequency in anesthetized rat, has been published only for obidoxime and K112 (10.6, 10.4), however, qualitatively similar effects could be expected from other reactivators as well. Once again, both the effect on the AChE and that on the M2 mAChRs appeared. Interestingly, it occurred the other way around than in the bladder, i.e. at lower doses the M2 mAChRs was affected and at higher doses the AChE. Tentatively, it indicates the *in-vivo* affinity of the oximes: M2>AChE>M3. The effect on AChE could possibly be observed since the stimulation of vagal nerve leads to the release of endogenous ACh, which is not broken down by inhibited AChE. In the case of K112 (10.4) a clear dose-dependent antimuscarinic effect was observed. However, no effect was observed on AChE. It might be explained by different experimental conditions and higher affinity of K112 to the mAChRs (data not shown); at 5 Hz stimulation instead of 10Hz, less acetylcholine is released and by that, it is harder to overcome the K112 antimuscarinic blockade at the lower frequency. In other words, the enzyme blockade does not overcome the muscarinic blockade (which might occur at larger doses than those examined). Other reactivators have also been examined and (unpublished) results show a similar progress like that in the case of obidoxime, supporting the idea of a dual effect of the reactivators. It must be said, that in case of the trimedoxime´s effect on bladder (10.3), this dual effect was not observed. This could be explained by comparable effects on AChE and on the muscarinic receptors, which counteraction leveled out the result seen in the composite response. The same type of *in-vitro* experiments but using atropine instead, showed, as could be expected, only the antimuscarinic action. However, a notably higher potency was observed.

The interaction of oximes with AChE was quantified by using the spectrophotometrical Ellman´s method (10.1-10.7). The potency to inhibit AChE occurred in the following order: HI-6>K027>K203>Obidoxime. Interestingly, this was in fact *vice versa* than the affinity of the same oximes to mAChRs. Tentatively, it may be concluded that the stronger AChE inhibitor, the less potent binding antagonist. On the other hand, we must take into consideration, that it is only relatively few observations in each group investigated in this way.

In-vivo observations on nicotinic effects (10.8) showed clearcut dose-dependent reductions in nerve-evoked twitches, which indicate a blockade of nicotinic receptors at the neuromuscular endplate. These effects could be observed at both 1 Hz and 5 Hz. At 5 Hz stimulation, smaller inhibitory effects appeared, probably due to a larger availability of ACh at this frequency. Even though no statistical significance appeared, still clear dose-dependent effect occurred. Concerning the individual compounds, only small differences in potency appeared. If any difference in potency occurred between the various oximes, it was somewhat less for K027. It should stressed that spontaneous fading of twitches may occur during a repetitive stimulation, probably due to changed sensitivity of the perceptual receptors [66]. However, a decrease by 23% was reported after delivery of 3000 stimuli. In our study, only about 1000 stimuli were delivered and the reduction observed was up to 50 %. Nevertheless,

the current results indicate a direct antagonistic effect of oxime reativators on the nAChRs. To the best of my knowledge, this is the first *in-vivo* report that oximes inhibit nicotinic receptors.

Interestingly, in the case of neuromuscular transmission, no effect on AChE was noticed, as in case of the muscarinic *in-vivo* examination. It must be considered that the physiological processes at central and neuromuscular synapsis differ. The AChE due to a posttranslational processes occur in different molecular forms, which has the same activity but differ in the affinity to AChE inhibitors [67]. Moreover, the approximate maximum response to autonomic stimulation occurs in the range 15 – 40 Hz. The corresponding interval to maximum responses to motoric stimulation occurs above 40 Hz (40 – 70 Hz or even higher). The quantal theory of ACh release approximates the release of 1000 molecules of ACh per quantum in the CNS and 6000-10000 per quantum in the neuromuscular junction [68]. In the current experiments, 10 Hz was applied for the vagal stimulation and only 1 and 5 Hz for neuromuscular stimulation. One explanation is that oximes have a more pronounced antagonistic effect on the nAChRs than on the mAChRs in relation to the AChEi effect. On the other hand, it has been shown that H-oxime has lower affinity to the nAChRs than on the mAChRs [56]. However, these two situations are somewhat incomparable and various theories are at hand concerning the effect on AChE, but the antagonistic effects on nAChRs and on the mAChRs are doubtless.

The antagonistic effect on the nAChRs has been also confirmed by using *in-vitro* patch-clamp technique (10.8). The results showed antagonistic activity of all tested compounds on the muscle type of nAChRs (IC_{50} in the range of hundreds of micromoles). Once again, apparent lower potency of K027 has been observed. Comparing this efficacy with *in-vitro* muscarinic examinations, the potency on the mAChRs is approximately by one fold higher. This is very well in agreement with previous observations [56].

In order to describe complex mechanisms of action of the reactivators, the interaction of oximes with the HACU transport system and their effect on membrane fluidity was investigated. This system is a key regulatory step (rate-limiting) in the synthesis of new ACh. Standard oxime reactivators (not published) and K112 (published, 10.4) were exerted to this system. In general, the results showed that oxime reactivators are able to interact with this system at low (K112, trimedoxime) or high concentrations (HI-6, obidoxime). In the first group the direct inhibitory effect on (3H)-choline reuptake was observed at low concentrations (10 μM). However, in the second group, a higher concentration (50μM) influenced the transport of (3H)-hemicholinium-3 (competitive, reversible, non-permeant ligand of the transport system). The inhibition of HACU is probably due to a direct interaction with the hemicholinium-sensitive carrier, since the effect on the anisotropy of the membrane is less likely to occur under the present conditions. Furthermore, inhibition was connected with changes in the affinity rather than with changes in the number of binding sites. These results exhibit strong evidence for other mechanisms being involved in the release of ACh. Furthermore, oxime reactivators are likely to influence them as well.

It is well known that results from *in-vitro* examinations do not always correlate with *in-vivo* findings [52, 56]. E.g. HI-6 can handle an *in-vivo* tabun poisoning [69], whereas it *in-*

vitro shows only a minute reactivation potency [70]. In our study, we observed a similar situation. HI-6, being less effective in the *in-vitro* testing, showed considerably higher potency when examined in the whole (isolated) organ or *in-vivo*. This supports the hypothesis that oxime reactivators dispose very complex mechanisms of action and in case of HI-6 it may explain its versatility and broad spectrum usage.

In conclusion, by using various *in-vitro* and *in-vivo* techniques, previous hypotheses are confirmed. Also, the thesis reveals new mechanisms in the antagonistic effect of oxime reactivators on the muscarinic and nicotinic receptors. This effect is likely to be of great significance in the treatment of OP poisoning, where the relative ACh excess can be counteracted by the blocking properties of oximes on cholinergic receptors. The comparison of the *in-vitro* data, showed the oximes to exert higher efficacy on the muscarinic than on the AChE. However, this effect seemed to be subtype specific since the antagonistic M3 effect was lower (M2>AChE>M3). Also, and importantly, the antagonistic muscarinic effect was larger than that on nicotinic receptors. Furthermore, in comparison with other drug used in the treatment, the classical antidote atropine showed a much higher muscarinic antagonism. Despite all of this, it is supposed that non-reactivation properties of oxime reactivators play a significant role in the treatment of OP poisoning. In the current publications, a dual effect on cholinergic receptors and on AChE at the same time is shown. This property is of course not in favor in the antidotal treatment. It should be stressed that this effect on AChE has not been observed in the nicotinic transmission. Since overstimulation of nicotinic is considered to be the cause of death in the OP poisoning, clear antinicotinic action is of advantage. To the best of my knowledge, this is the first time that it is shown that oximes inhibit nicotinic receptors *in-vivo*.

5 References

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6 Outputs:

6.1 Publications related to the thesis

1. Soukup O, Pohanka M, Tobin G, Jun D, Fusek J, Musilek K, et al. (2008). The effect of HI-6 on cholinesterases and on the cholinergic system of the rat bladder. Neuroendocrinology Letters 29: 759-762.

2. Soukup O, Proska J, Binder J, Karasova JZ, Tobin G, Jun D, et al. (2009). Methylacridinium and its Cholinergic Properties. Neurotoxicity Research 16: 372-377.

3. Soukup O, Holas O, Binder J, Killy K, Tobin G, Jun D, et al. (2010). The effect of trimedoxime on acetylcholinesterase and on the cholinergic system of the rat bladder. Journal of Applied Biomedicine 8: 87-92.

4. Soukup O, Kristofikova Z, Proska J, Tobin G, Patocka J, Marek J, et al. (2010). Novel acetylcholinesterase reactivator K112 and its cholinergic properties. Biomedicine et Pharmacotherapy 64: 541-545.

5. Soukup O, Tobin G, Kumar UK, Binder J, Proska J, Jun D, et al. (2010). Interaction of Nerve Agent Antidotes with Cholinergic Systems. Current Medicinal Chemistry 17: 1708-1718.

6. Soukup O, Tobin G, Kumar UK, Jun D, Fusek J and Kuca K (2010). Characterization of the anticholinergic properties of obidoxime; functional examinations of the rat atria and the urinary bladder. Toxicol Mech Methods 20: 428-433.

7. Soukup O, Kumar UK, Proska J, Bratova L, Adem A, Jun D, et al. The effect of oxime reactivators on the muscarinic receptors; functional and binding examination. Environmental Toxicology and Pharmacology (in press). DOI:10.1016/j.etap.2011.01.003

8. Soukup O, Krusek J, Kaniakova M, Kumar UK, Oz M, Jun D, et al. Oxime reactivators - in vivo and in vitro effects on nicotinic receptors. Physiological Research (accepted for publication on the 21th of February 2011, see appendix 10.8)

6.2 Other publications

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8 Summary in English

Currently, organophosphorus (OP) poisoning is a threat particularly in war conflicts in connection with terrorism or a poisoning caused by OP pesticides in agriculture. The poisoning is caused by inhibition of the enzyme acetylcholinesterase (AChE), which, under physiological conditions, cleaves acetylcholine (ACh) and regulates the transmission of nerve signals. The inhibition of AChE leads to excessive stimulation of cholinergic receptors and clinically it manifests as so-called cholinergic syndrome. According to the structures affected, symptoms are divided into nicotinic, muscarinic and central. Organophosphates are lethal compounds, when death is caused by suffocation. Current treatment is mainly based on the application of anticholinergics (atropine) and /or on the application of AChE oxime reactivators (HI-6, obidoxime, pralidoxime etc.) Atropine inhibits excessive neural transmission by blocking muscarinic receptors. However, it is only symptomatic cure not causal. Whereas oximes, which represent a causal therapy, are able to unbind an OP compound from the AChE and restore its splitting function.

Using reactivators has two major drawbacks; there is no universal reactivator against all types of OP compounds (soman, sarin, paraoxon etc.) and reactivation is possible only within a certain time after the exposure ("aging"). Based on these limiting factors, it is essential to keep searching for a universal oxime or apply different therapeutic approaches.

One possible approach is a deeper examination of the reactivators´ mechanism of action since they have a very complex effect on the whole cholinergic system. In particular, their ability to influence the nervous signal transmission at the receptor level.

The aim of this dissertation thesis is to study the cholinergic effects of oxime reactivators (especially their effect on the muscarinic and nicotinic receptors) and the significance of non-reactivating properties in the treatment of organophosphate poisoning. We selected two commonly used reactivators (HI-6 and obidoxime) and 2 newly synthesized reactivators (K027 and K203) for this purpose. These 4 compounds were studied by using various techniques involving *in-vitro* and *in-vivo* experiments. Specifically, reactivators' affinity for muscarinic receptors, effect on signal transduction, functional experiments on the tissue samples (isolated heart atrium, urinary bladder in the organ bath) and on the whole body (impact on rat heart rate). Nicotinic effects were also studied using *in-vitro* (cell patch-clamp) and *in-vivo* (rat neuromuscular effects) methods. Other experiments were performed in order to describe a direct effect on AChE or on the choline re-uptake as the other cholinergic structures that may be affected by reactivators.

The experimental data showed that AChE reactivators have a very complex mechanism of action. They exert the inhibitory effect on AChE with the efficacy at least 3-fold lower than the commonly used central inhibitors of AChE. Furthermore, they display inhibitory effect on the high-affinity choline uptake transporter (HACU), which is a key-regulatory step in the ACh synthesis *de novo.* I*n-vitro* and *in-vivo* experiments comprehensively proved inhibitory effects of studied oximes on nicotinic receptors. Although, the IC_{50} values were in the hundreds of micromoles, even a weak inhibition may speak in

favor of the treatment with reactivators. Moreover, atropine does not affect nicotinic receptors at all and unfortunately nicotinic symptoms are usually responsible for the death of a victim. Muscarinic antagonism has also been verified by both *in-vitro* and *in-vivo* experiments. Although, the mechanism of binding has not been satisfactorily explained a weak inhibition was observed (atropine in all experiments possesses at least 3-fold higher efficacy). However, even a weak inhibition may play an important role in the treatment of OP poisoning, in particular, in the case of "aging".

Focusing on individual reactivators- HI-6, obidoxime, and K203 usually demonstrated a higher inhibitory efficiency than K027. Surprisingly, HI-6, that exerted the lowest affinity for muscarinic receptors, showed notably higher efficiency in more complex systems (the tissue level, the whole organism level). This fact suggests other mechanisms, than performed in this study, may be involved (e.g. the effect on presynaptic receptor, synthesis or the release of ACh). Moreover, it also explains why HI-6 seems to be currently the most effective reactivator with the broadest spectrum of action.

Review article and results from this dissertation thesis were published in Czech and international scientific journals (8x first-author papers, 7x IF). Furthermore, results were also presented at Czech and international conferences in the form of lectures or poster presentations.

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9 Shrnutí v českém jazyce

Otrava organofosforovými (OF) sloučeninami je v současné době hrozbou zejména ve válečných konfliktech, ve spojení s terorismem či jako otravy způsobené v zemědělství OF pesticidy. Otrava je způsobena inhibicí enzymu acetylcholinesterázy (AChE), který za fyziologických podmínek štěpí acetylcholin (ACh) a reguluje tak přenos nervového signálu. V případě inhibice AChE dochází k nadměrné stimulaci cholinergních receptorů a klinicky se manifestuje jako tzv. cholinergní syndrom. Dle zasažených struktur se příznaky dělí na nikotinové, muskarinové či centrální. Jedná se o, dle dávky a doby expozice, letální látky, kdy smrt nastává udušením. Současné léčba sestává z aplikace anticholinergik (atropin), které blokují nadměrný nervový přenos inhibicí zejména muskarinových receptorů a/nebo z aplikace reaktivátorů AChE (HI-6, obidoxim, pralidoxim etc.), které jsou schopny vyvázat OF látku z vazby na AChE a tím obnovit její štěpící funci. Tato léčba je však limitována tím, že neexistuje univerzální reaktivátor, který je schopný vyvázat libovolnou nervově paralytickou látku (soman, sarin, paraoxon etc.), a tím, že reaktivace AChE je možná pouze do určité doby po expozici („aging“). Na základě těchto limitujících faktorů jsou vyvíjeny další universálnější reaktivátory či jsou vyhledávány jiné terapeutické přístupy. Tím spíš, že léčba atropinem je pouze léčba symptomatická nikoliv kauzální.

Jedním z takových přístupů je hlubší prozkoumání mechanismu účinku reaktivátorů, protože se jedná o látky s velmi komplexním účinkem na celý cholinergní systém. Zejména jejich schopnost ovlivňovat přímo přenos signálu na receptorové úrovni (mechanismus podobný receptorové blokádě atropinem).

Cílem této dizertační práce je prostudovat cholinergní účinky oximových reaktivátorů (zejména jejich účinek na muskarinové a nikotinové receptory) a posoudit přínos v terapii otrav organofosfáty u vlastností, které nemají nic společného s reaktivací AChE.

K tomuto účelu byly vybrány 2 běžně používané reaktivátory (HI-6 a obidoxim) a 2 nově syntetizované reaktivátory (K027 a K203). Tyto 4 látky byly studovány za využití různých technik v *in-vitro* i *in-vivo* experimentech. Konkrétně se jednalo o afinitu reaktivátorů k muskarinovým receptorům, jejich ovlivnění transdukce signálu, funkční experimenty jak na úrovni orgánů (izolované srdeční síně, močový mechýř v orgánové lázni) tak i na úrovni celého organismu (ovlivnění srdeční frekvence potkana). Nikotinické účinky byly studovány taktéž *in-vitro* (patch-clamp) i *in-vivo* (ovlivnění nervosvalového přenosu potkana). Další experimenty zabývající se přímým ovlivněním AChE reaktivátory či jejich vliv na re-uptake cholinu byly provedeny za cílem obsáhnout i další cholinergní struktury, které mohou být reaktivátory ovlivněny.

Výsledky experimentů ukázaly, že reaktivátory AChE vykazují velice komplexní mechanismus účinku. Byl prokázán inhibiční účinek na AChE v řádech minimálně 1000x menších než běžně používané inhibitory AChE při léčbě Alzheimerovy demence. Dále pak byl prokázán inhibiční účinek na vysokoafinitní transport cholinu (HACU), který je rozhodující pro syntézu ACh *de novo*. Účinek na nikotinové receptory, byl z *in-vitro* i *in-vivo* experimentů shledán jako inhibiční. Ačkoliv se hodnoty IC_{50} pohybovaly v řádech stovek mikromolů, může tato slabá inhibice hovořit ve prospěch léčby reaktivátory tím spíš, že atropin nemá na nikotinové receptory prakticky žádný vliv. Muskarinový antagonismus byl

taktéž ověřen *in-vitro* i *in-vivo* pokusy. Ačkoliv mechanismus vazby nebyl uspokojivě vysvětlen, jedná se opět o slabé inhibitory (atropin ve všech experimentech byl minimálně 1000x více účinný), avšak i tato slabá inhibice může hrát v léčbě otrav OF důležitou roli. Zejména v případě uplynutí doby, po kterou je AChE reaktivovatelná (po „agingu“).

Pokud se jedná o jednotlivé reaktivátory, většinou HI-6, obidoxime a K203 vykazovaly větší inhibiční efektivitu; K027 většinou menší. Překvapivě, látka HI-6 s nejnižší afinitou k muskarinovým receptorům vykazovala v komplexnějších systémech (orgán, organismus) vysokou účinnost, což nasvědčuje tomu, že i další než studované mechanismy mohou být zastoupeny (např. ovlivnění presynaptických receptorů, syntézy či uvolňovaní ACh). Zároveň dosažené výsledky vysvětlují to, proč se HI-6 v současné době jeví jako nejúčinnější reaktivátor s nejširším spektrem účinku.

Rešerše a výsledky obsažené v této dizertační práci byly publikovány v českých i zahraničních odborných časopisech (8 prvoautorových prací, 7x s IF) a na českých i zahraničních konferencích v rámci přednášek či formou posterových sdělení.

10 Appendices: