# GÖTEBORG UNIVERSITY
SAHLGRENSKA ACADEMY

Department of Pharmacology

&

# UNIVERZITA KARLOVA V PRAZE

FARMACEUTICKÁ FAKULTA
V HRADCI KRÁLOVÉ

Katedra farmakologie


## The effect of acetylcholinesterases reactivators on the cholinesterases and cholinoreceptors

## Vliv reaktivátorů acetylcholinesterázy na cholinesterázy a cholinoreceptory

Diploma thesis


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Hradec Králové 2010


Lucie Brátová

I declare that my diploma thesis is my original publication which I have written by myself. All used literature and other sources are named in References and cited properly.

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# Abstrakt

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Kandidát: Lucie Brátová
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Název diplomové práce: Vliv reaktivátorů acetylcholinesterázy na cholinesterázy a cholinoreceptory

Diplomová práce se zabývá efektem oximů na muskarinových receptorech. V první půlce teoretické části je popsán autonomní nervový systém, hlavní receptory a funkce acetylcholinesterázy. Druhá půlka shrnuje důležité nervové plyny, otravu organofosfáty a její léčbu. Závěrem představuje oximy a jejich mechanismus účinku.
Praktická část popisuje metodiku experimentů na potkaních srdečních síních. Jak *in vitro,* tak *in vivo* studie prokázaly efekt oximů na M2 receptory a jejich antimuskarinové vlastnosti. *In vivo* experimenty byly zaměřeny na protichůdné účinky oximů – inhibice AChE vysokými koncentracemi oximu a inhibice muskarinových receptorů nízkými koncentracemi toho samého oximu.

# Abstrakt

Charles University in Prague
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Department of Pharmacology

Candidate: Lucie Brátová
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Title of Diploma Thesis: The effect of acetylcholinesterases reactivators on the cholinesterases and cholinoreceptors

This thesis is concerned with the effects of oximes on the muscarinic receptors. In the first half of the theoretical part there is a description of the autonomic nervous system, main receptors and action of acetylcholinesterase (AChE). The second part summarizes important nerve gases, organophosphate poisoning and its treatment and finally introduces oximes and their mechanisms of action.
The practical part shows the metodology of experiments on the atria tissues in the rat. Both *in vitro* and *in vivo* procedures demonstrated effect of oximes on the M2 receptors and their antimuscarinic properties. The *in vivo* studies were focused on the two contradictory effects – the inhibition of AChE and the inhibition of muscarinic receptors by the very same compound.

# I. Introduction

The increased international concern about the threat of military and terroristic use of nerve agents and the fact that oximes are not always very effective as reactivators and none of them can be regarded as a broadspectrum antidote, those are the main reasons why futher research and investigation are being conducted. The experiments confirmed a hypothesis that not only a well-known cholinesterase reactivation activity of oximes but also their multiple effects - on muscarinic and nicotinic receptors – may play a key role in their life-saving effects. This has made the research field much wider.

# II. Review of literature

## *A. The nervous system*

The nervous system can be divided into two anatomical parts: the central nervous system (CNS), which is composed of the brain and spinal cord, and the peripheral nervous system, which includes the rest of the neurons located outside the brain and spinal cord. We can subdivide those nerves into efferent division, the neurons that carry signals to the peripheral tissues, and the afferent division, the neurons that bring information from the sensory receptors at the periphery to the CNS. Efferent fibers are further divide into two functional groups – somatic and autonomic, also called visceral or vegetative, system. The somatic efferent neurons are involved in the voluntary control of function such as contraction of the skeletal muscle. On contrary to autonomic system, single myelinated motor neuron, originating in the CNS, travels directly to skeletal muscle without the mediation of ganglia. The efferent nerves of the involuntary system supply all innervated structures of the body except skeletal muscle, and this is provided by autonomic system (Howland, Mycek et al. 2006).

Autonomic nerve fibers affect the target organs by a two-neuron pathway. The cell bodies of the peripheral neurons are grouped together to form ganglia. The first neuron, preganglionic, passing from the CNS to the ganglia synapses with a second (postganglionic) neuron that innervates the target organ. The preganglionic fibers are slow-conducting (class B and C) which release acetylcholine (Nachon, Asojo et al.). The final motor neurons from the ganglia to the tissues, the postganglionic fibers, are also slow-conducting, unmyelinated class C fibers (Golan 2005; Petersen 2007).

Autonomic system is classically divided into two subsystems: sympathetic nervous system mediated by noradrenalin and parasympathetic nervous system mediated by acetylcholine.

## 1. “Fight or flight” response

The sympathetic nervous system is also known as the thoracolumbar system, because its preganglionic fibers arise from the first thoracic segment to the second or third lumbar segment of the spinal cord (Golan 2005). Postganglionic (adrenergic) fibers release the neurotransmitter noradrenalin. The adrenal medullae release the hormone adrenaline, and also some noradrenalin, into the blood stream rather than directly on to effectors’ cells. Some sympathetic nerves that innervate the blood vessels of muscles, sweat glands and hair follicles in the skin release acetylcholine instead of noradrenalin (Petersen 2007). Sympathetic division is to some degree continually active but the main property is to cope with stressful situations such as trauma, fear, hypoglycemia, cold or exercise. The effect of sympathetic output is to increase the heart rate and the blood pressure, to mobilize energy stored in body, and to increase blood flow to skeletal muscle and the heart while reducing it from the skin and internal organs (Howland, Mycek et al. 2006).

## 2. “Rest and digest” response

The preganglionic neurons of the parasympathetic system come from both brainstem and sacral segments of the spinal cord – thus, the parasympathetic system is also called the craniosacral system. There are no paravertebral ganglia in this system, nearly all of the parasympathetic ganglia lie in or near the organs they innervate. It usually acts to oppose or balance the action of the sympathetic division and is generally dominant over the sympathetic system in “rest and digest” situations. It is necessary to say that parasympathetic system never discharges in all innervated organs; instead, discrete parasympathetic fibers are activated separately in order to prevent massive, undesirable unpleasant symptoms and to affect specific organs, such as stomach or eye (Howland, Mycek et al. 2006; Petersen 2007).

### 3. Non-adrenergic non-cholinergic (NANC) neurones

There are many autonomically innervated tissues (e.g. in the cardiovascular, respiratory and urinary systems), where neurotransmitters other than noradrenalin and acetylcholine are released from postganglionic motor neurons to modify the activity of effector organs. There is an evidence that these compounds (e.g. peptides, ATP, NO or opioids) are released either along with the classical transmitter as a co-transmitters, primary transmitters, or as neuromodulators (Petersen 2007).

## *B. Cholinergic system*

The cellular actions of acetylcholine are mediated by two structurally diverse families of membrane-bounded proteins, the nicotinic (nAChR) and the muscarinic (mAChR) receptors. These receptors, both of which are composed of multiple subtypes, are expressed pre- and postsynaptically and have discrete distributions throughout the central and peripheral nervous systems.

**Table 1:** Muscarinic acetylcholine receptor subtypes (Rang and Dale 2007)

| **Characteristic** | **$M_1$ ("neural")** | **$M_2$ ("cardiac")** | **$M_3$ ("glandular/smooth muscle")** | **$M_4$** | **$M_5$** |
|---|---|---|---|---|---|
| **Main locations** | CNS: cortex, hippocampus<br>Glands: gastric, salivary, etc. | Heart: atria<br>Smooth muscle: GI tract<br>CNS: widely distributed | Exocrine glands: gastric, salivary, etc.<br>Smooth muscle: GI tract, eye<br>Blood vessels: endothelium | ? Lung CNS: cortex, striatum | CNS: very localized expression in substantia nigra<br>Salivary glands<br>Iris/ciliary muscle |
| **Cellular response** | ↑ $IP_3$, DAG<br>Depolarization<br>Excitation<br>↑ $K^+$ conductance | ↓ cAMP Inhibition<br>↓ $Ca^{2+}$ conductance<br>↑ $K^+$ conductance | ↑ $IP_3$ Stimulation<br>↑ $(Ca^{2+})_i$ | As $M_2$ | As $M_3$ |
| **Functional response** | CNS excitation (?memory)<br>Gastric secretion | Cardiac inhibition<br>Neural inhibition<br>Central muscarinic effects, e.g. tremor, hypothermia | Gastric, salivary secretion<br>GI smooth muscle contraction<br>Ocular accommodation<br>Vasodilatation | Enhanced locomotion | Not known |

**Table 2:** Nicotinic acetylcholine receptor subtype (Rang and Dale 2007)

| **Characteristic** | **Muscle type** | **Ganglion type** | **CNS type** |
|---|---|---|---|
| **Main synaptic location** | Skeletal neuromuscular junction: mainly postsynaptic | Autonomic ganglia: mainly postsynaptic | Many brain regions: pre- and postsynaptic |
| **Membrane response** | Excitatory<br>Increased cation permeability (mainly $Na^+$, $K^+$) | Excitatory<br>Increased cation permeability (mainly $Na^+$, $K^+$) | Pre- and postsynaptic excitation<br>Increased $Ca^{2+}$ permeability |

## 1. Nicotinic receptors

All nicotinic receptors (Table 2) are pentameric structures which function as ligand-gated ion channels and are responsible for fast excitatory synaptic transmission. They fall into three main classes: the muscle, ganglionic and CNS types. Muscle receptors can be found at the skeletal neuromuscular junction, ganglionic receptors are responsible for transmission at sympathetic and parasympathetic ganglia, CNS-type receptors are widespread in the brain and are quite heterogeneous. They differ in molecular structure and thus they have distinct pharmacological properties (Rang and Dale 2007).

The five subunits that form the receptor-channel complex are similar in structure. The α subunits possess two adjacent cysteines essential for acetylcholine binding, whereas the non-α referred to as β, γ, ε, or δ do not (Dani and Bertrand 2007). But there might be several new nAChR subunit genes still uncovered in the human genome.

A lateral cross section of the nAChR displays an extracellular water-filled vestibule and extends from the membrane surface into the synaptic cleft (Dani and Bertrand 2007).

## 2. Muscarinic receptors

The muscarinic receptors belong to the family of G-protein-coupled receptors. The G proteins, consisting of one α-, β- and γ-subunit, are subdivided into $G_s$, $G_{i/o}$, $G_q$ and $G_{12}$ depending on the primary sequence homology of their α-subunits. The muscarinic receptor subtypes couple differentially to the G proteins, and the subunits of G proteins activate distinct cellular pathways (Giglio and Tobin 2009).

The muscarinic receptors are considered to comprise five subtypes - muscarinic M1, M2, M3, M4 and M5 receptors, but only four have been distinguished functionally and pharmacologically (Table 1). The pharmacological classification of these receptor types relies on the limited selectivity of certain agonists and antagonists that can distinguish between them (Rang and Dale 2007).

**M1-receptors** are found mainly on CNS and peripheral neurons, gastric parietal cells and significant levels have been reported also in salivary glands (Tobin, Giglio et al. 2002). They mediate excitatory effects, for example the slow muscarinic excitation

mediated by acetylcholine in sympathetic ganglia and central neurons. This excitation is produced by a decrease in $K^+$ conductance, which causes membrane depolarization. Deficiency of this kind of acetylcholine-mediated effect in the brain is possibly associated with dementia. M1-receptors are also involved in the increase of gastric acid secretion following vagal stimulation (Tobin, Giglio et al. 2009).

**M2-receptors** occur in the heart, and also on the presynaptic terminals of peripheral and central neurons. They exert inhibitory effects, mainly by increasing $K^+$ conductance and by inhibiting calcium channels (Rang and Dale 2007). M2-receptor activation is responsible for the vagal inhibition of the heart, as well as the presynaptic inhibition in the CNS and periphery.

**M3-receptors** produce mainly excitatory effects, i.e., stimulation of glandular secretions (salivary, bronchial, sweat, etc.) and contraction of visceral smooth muscle. M3-receptors also mediate relaxation (mainly vascular) of smooth muscle, which results from the release of nitric oxide from neighbouring endothelial cells (Golan 2005).

The odd-numbered members of the group (M1, M3, M5) act through the inositol phosphate pathway, while the even-numbered receptors (M2, M4) act by inhibiting adenylate cyclase and thus reducing intracellular cAMP (Rang and Dale 2007).

The subtypes of the receptor population interact on neuronal as well as on non-neuronal cells in regulation of autonomic responses.

## a) Neuronal muscarinic receptors

The prejunctional inhibitory muscarinic receptor was for a long time considered to be the M2 subtype. On the other hand, binding studies have indicated the best correlation to the muscarinic M4 receptor and not the muscarinic M2 receptor in some organs. Therefore, there are two distinct subtypes on prejunctional terminals in several peripheral tissues, including smooth muscle and glandular tissue.

## b) Non-neuronal muscarinic receptors

It has been proved that muscarinic M3 receptors mediate most postjunctional effects. However, postjunctional muscarinic M2 receptors also occur, commonly co-localised with the muscarinic M3 receptor. Studies in several species, including man, indicate synergistic effects of M2 and M3 receptors in controlling smooth muscle contraction (Tobin, Giglio et al. 2009).

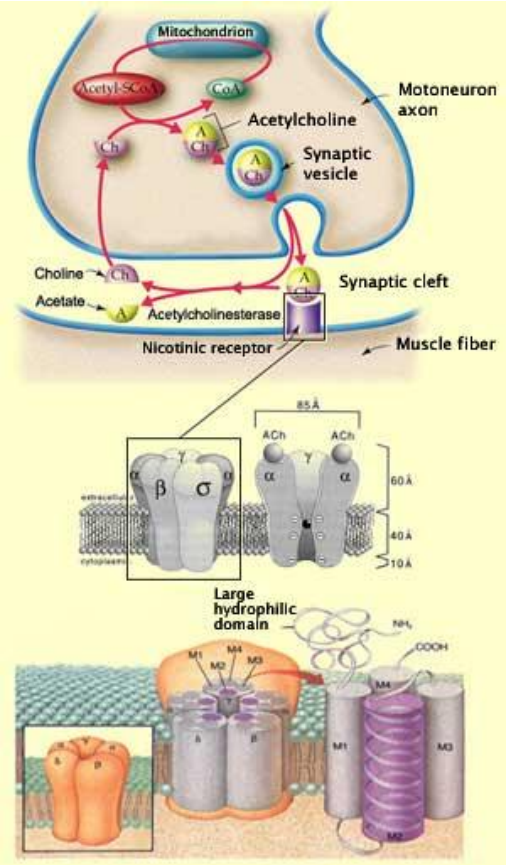



**Figure 1:** Adapted from
http://thebrain.mcgill.ca/flash/a/a_06/a_06_m/a_06_m_mou/a_06_m_mou.html
Figure shows the neuromuscular junction, how the acetylcholine is synthetized, stored, released and broken down. It also describes the structure of the nicotinic receptor.

## *C. Cholinoesterases (ChEs)*

There are two distinct ChEs for transmitter hydrolysis: acetylcholinesterase (EC 3.1.1.7, AChE) and butyrylcholinesterase (EC. 3.1.1.8, BuChE) (Adler, Manley et al. 2004).

While AChE is found in cholinergic neurons, it is distributed more widely than cholinergic synapses. It is highly concentrated at the postsynaptic end-plate of the neuromuscular junction. Furthermore, it also occurs in red blood cells. BuChE is synthesized primarily in the liver and is found in liver and plasma. AChE and BuChE can be distinguished by the relative rates of acetylcholine (ACh) and butyrylcholine hydrolysis, by the affinity to various substrates and by effects of selective inhibitors (Goodman and Brunton 2008). AChE is quite specific for ACh and closely related esters such as methacholine. BuChE has broader substrate specificity than AChE but is not particularly associated with cholinergic synapses, and its physiological function is unclear.

Both consist of globular catalytic subunits, which are linked to collagen-like proteins or to glycolipids to the cell membrane or the basement membrane at various sites, including cholinergic synapses (and erythrocyte membrane, where the function of the enzyme is still unknown) (Rang and Dale 2007).

### 1. Acetylcholinesterase

AChE is capable of hydrolyzing about 4 x $10^5$ molecules of ACh per enzyme molecule per minute, and so its turnover time of 150 microseconds makes it one of the most efficient hydrolytic enzymes known (Golan 2005). Inhibition of AChE results in a progressive accumulation of ACh, especially during periods of repetitive stimulation (Adler, Manley et al. 2004). Cholinesterases are characterized as the main enzymes involved in the toxic effect of organophosphorus nerve agents (Bajgar 2004).

AChE exists in two general classes of molecular forms: simple homomeric oligomers of catalitic subunits (i.e., monomers, dimers, and tetramers) and heteromeric associations of catalytic subunits with structural subunits (Perrier, Cousin et al. 2000). The 3-D structures of AChE show the active center to be almost centrosymmetric to each subunit

and is located at the base of a narrow gorge about 20 Å in depth (Sussman, Harel et al. 1991).

## 2. Effects of anticholinesterase drugs

Cholinesterase inhibitors affect peripheral as well as central cholinergic synapses. The characteristic pharmacological effects of the anti-ChE agents are primarily due to the prevention of hydrolysis of ACh by AChE at the sites of cholinergic transmission.

### a) Effects on autonomic cholinergic synapses

These mainly reflect an enhancement of ACh activity at parasympathetic postganglionic synapses (i.e., increased secretion from salivary, lacrimal, bronchial and gastrointestinal glands; increased peristaltic activity; bronchial constriction; bradycardia and hypotension; pupillary constriction; fixation of acommodation for near vision; fall in intraocular pressure).

Acute anticholinesterase poisoning causes severe bradycardia, hypotension and difficulty in breathing. In combination with a depolarising neuromuscular block, and central effects, the result may be fatal.

### b) Effects on the neuromuscular junction

Anticholinesterase agents increase the twitch tension of a muscle stimulated via its motor nerve. Inhibiting AChE gives the ACh molecules a greater chance of finding a vacant receptor before being destroyed. In myastenia gravis, transmission fails because there are too few ACh receptors, and cholinesterase inhibition improves transmission.

In large doses, such as in organophoshourus poisoning, anticholinesterase drugs initially cause twitching of a muscle. Later, paralysis may occur due to depolarisation block, which is associated with the cumulation of ACh in the plasma and tissue fluids (Rang and Dale 2007).

### c) Neurotoxicity of organophosphates

Tertiary compounds, such as physostigmine, and the non-polar organophosphates penetrate the blood-brain barrier freely and affect the brain. The result is an initial excitation, which can result in convulsions, followed by depression, which can cause unconsciousness and respiratory failure. These central effects result mainly from the activation of muscarinic receptors (mAChRs) (Rang and Dale 2007), and are antagonised by atropine.

Many organophosphates can cause a severe type of peripheral nerve demyelination, leading to progressive weakness and sensory loss. Similar effect could be presumed in the CNS.

## *D. Organophosphorus Compounds*

Shortly before and during World War II, a new class of highly toxic chemicals, the organophosphates, was developed first as agricultural insecticides and later as potential chemical warfare agents. It was Gerhard Schrader who first defined the structural requirements for anti-ChE activity after synthesizing approximately 2000 compounds (Goodman and Brunton 2008).

### 1. Insecticides

One of the compounds in the early series, *parathion* (a phosphorothionate) later became the most widely used insecticide of this class. The reason is because of its low volatility and stability in aqueous solution. Parathion itself is inactive in inhibiting AChE in vitro; paraoxon is the active metabolite. Other insecticides possessing the phosphorothioate structure have been widely employed for home, garden and agricultural use. These include *diazinon* and *chlorpyrifos* (Goodman and Brunton 2008).

*Malathion*, also possessing thionophosphorus bond, is a precursor as well. The detoxication reaction is much more rapid in mammals and birds than in insects. In recent years, malathion has been employed in aerial spraying of relatively populous

areas for control of Mediterranean fruit flies and mosquitoes that harbor and transmit viruses harmful to human beings, such as the West Nile encephalitis virus. Evidence of acute toxicity arises only with suicide attempts or deliberate poisoning (Bardin, van Eeden et al. 1994).

## 2. Nerve agents (NAs)

The first organophosphorus nerve agents, tabun (GA) and sarin (GB), were developed in the 1930s by Gerhard Schrader, later in 1944 was synthetized even more toxic soman (McNamara, Pulido-Rios et al. 2009). Together with VX, developed after World War II in the United Kingdom, these compounds have emerged as the major nerve agents (Jokanovic 2009). Simple and inexpensive to make, easy to disperse, difficult to detect, feared by the public, and with a potential lethality to kill hundreds in one attack, NAs are nearly the ideal terrorist weapon. Insidious employment of these agents has occurred in warfare and terrorism attacks (e.g. Tokyo subway attack) (Nozaki, Aikawa et al. 1995).

Nerve agents can be divided into two groups – G-series agents (abbreviation of "German"): GA (tabun), GB (sarin), GD (soman), GF (cyclosarin) and V series agents (abbreviation of "venom"): VX and VR. The G agents are viscous colorless liquids of high volatility and inexpressive odor. V agents are colorless, susceptibly less volatile liquids of a high stability (Bajgar 2004).

The general formula for this class of cholinesterase inhibitors is presented in Figure 3. A great variety of substituents is possible: R1 and R2 may be alkyl, alkoxy, aryloxy, amido, thio, or other groups, and X, the leaving group, a conjugate base of a weak acid, is found as a halide, cyanide, thiocyanate, phenoxy, thiophenoxy, phosphate, thiocholine, or carboxylate group.

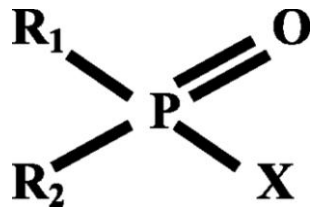



**Figure 3**

Tabun contains a cyanide group. Sarin and soman, which contain a fluorine substituent group, are methylphosphonofluoridate esters. These nerve agents contain a C–P bond that is almost unique in that it is not found in organophosphate pesticides. This C–P bond is very resistant to hydrolysis. VX contains a sulfur and is an alkylphosphonothiolate.

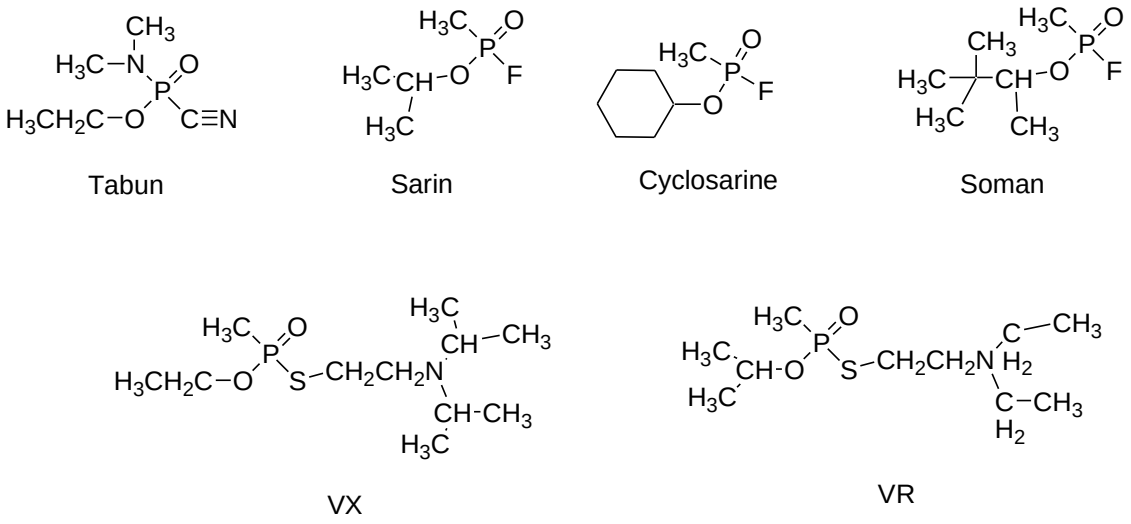



**Figure 4:** Organophosphorus nerve agents

Nerve agents react rapidly with a serine hydroxyl group in the active site of AChE with the formation of a phosphate or phosphonate ester. The phosphorylated enzyme regenerates very slowly leaving the enzyme inaccessible for its physiological substrate ACh. Both organophosphorus (OP) pesticides and nerve agents loose their acyl radicals when they react with AChE, ChE and carboxylesterase (CarbE). After binding to ChE,

the phosphoryl residues of soman, sarin, tabun and VX undergo an intramolecular rearrangement with subsequent loss of one phosphoryl group. This reaction is known as **aging** defined as non-enzymatic time-dependent loss of one alkyl group bound to the phosphorus which leads to a stable non-reactivatable form of phosphorylated AChE (Sidell and Groff 1974; Jokanovic 2009). Aging of nerve agents such as soman is very fast and occurs in a few minutes, while aging of pesticides such as paraoxon or ethyl-dichlorvos can take hours to days (Nachon, Asojo et al. 2005).

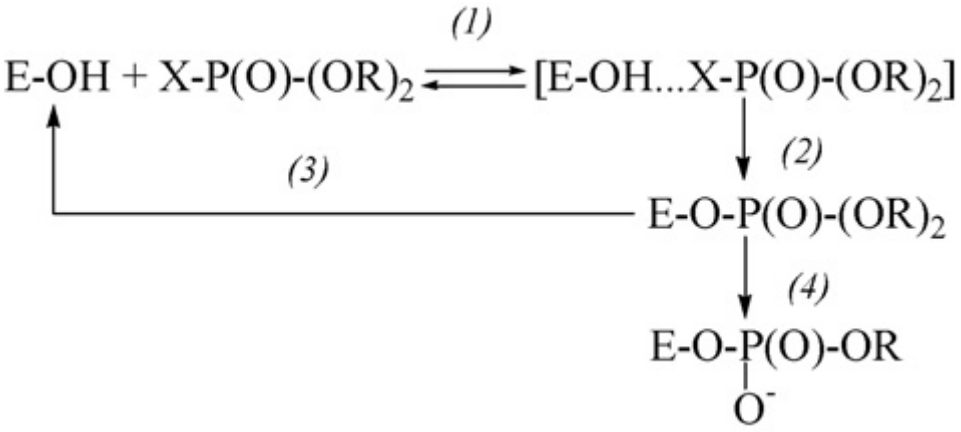



**Figure 5.** Interaction of esterases (E) (such as acetylcholinesterase) with organophosphorus compounds. Reaction 1 shows interaction of organophosphate molecule with the serine hydroxyl group at the active site of esterase via formation of an intermediate Michaelis–Menten complex leading to phosphorylated enzyme (Reaction 2). Reaction 3 is a spontaneous reactivation of inhibited esterase which occurs very slowly for most OP. Reaction 4, known as "aging", represents time-dependent loss of one alkyl group (R) bound to the phosphorus (Jokanovic 2009).

## a) Clinical signs and symptoms of nerve agent intoxication

The effects of acute intoxication by anti-ChE agents are manifested by muscarinic and nicotinic signs and symptoms and, except for compounds with extremely low lipid solubility, by signs referable to the CNS. The syndrom of NA intoxication is therefore a constellation of parasympathetic effects, neuromuscular junction (NMJ) function failure, and impairment of CNS function. In a roughly dose-dependent manner, this

translates primarily into miosis, blurred or dim vision with headache, tearing, bronchoconstriction, excessive upper and lower respiratory secretion, nausea with vomiting or diarrhea, fasciculations progressing to paralysis, altered mental status, loss of consciousness, seizures and apnea (Leikin, Thomas et al. 2002). Duration of the effects is determinated largely by the properties of the compound: its lipid solubility, whether or not it must be activated to form the oxon, the stability of the organophosphorus bond, and whether or not "aging" of the phosphorylated enzyme has occurred (Goodman and Brunton 2008). The particular symptoms and signs a patient displays are a product of the dose he has received and the route of exposure. Exposure may come via inhalation of vapor, absorption by the conjuctiva, absorption by the skin or mucous membranes, wound absorption, or absorption by the gut (Cannard 2006). In general, vapor inhalation is the most lethal route, largely because the dose can be so rapidly delivered to the systemic circulation and CNS. Death can ensue within seconds to minutes. However, because of lack of a depot effect following inhalation, if one can survive the initial 20-30 min of vapor exposure without respiratory failure, the chance of survival is very high (Nozaki, Aikawa et al. 1995).

Delayed symptoms appearing after one to four days are marked by persistent low blood cholinesterase and severe muscle weakness (Marrs 1993). A delayed neurotoxicity also may be evident after severe intoxication.

| Clinical severity<br><br>Symptom category | Mild | Moderate | Severe |
|---|---|---|---|
| **Autonomic (parasymathetic)** | Miosis, dim or blurred vision, eye pain or headache, tearing, rhinorrhea or sialorrhea, mild wheezing, local sweating (skin exposure) | Bronchorrhea, dyspnea, nausea/vomiting, abdominal cramps, incontinence, mild arrhythmias | Copious secretions, severe airway resistance, dangerous arrhythmias |
| **NMJ** | None | Mild weakness, fasciculations | Flaccid paralysis, apnea |
| **CNS** | None | Irritability, anxiety, impaired judgment or memory, slurred speech | Altered mental status, loss of consciousness, seizures, central apnea |

**Table 4:** Clinical signs and symptoms of nerve aget intoxication (Cannard 2006)

Clinical diagnosis is relatively simple and is based on medical history and circumstances of exposure as well as the presence of symptoms of poisoning. Confirmation of diagnosis can be made by measurement of red blood cell AChE activity or plasma ChE activity, which are accepted as biomarkers of exposure to OP compounds and carbamates (Jokanovic and Stojiljkovic 2006).

## b) Treatment of organophosphorus poisoning

### *(1) Prophylaxis*

Prophylaxis is an approach when an exposure to organophosphates is supposed. There are various options available.

(1) First and well known is an administration of inhibitors that are able to inhibit AChE reversibly and after recovery, normal AChE serves as the enzyme source. Examples of

such inhibitors are carbamates (pyridostigmine, physostigmine, aminostigmine). Pyridostigmine was introduced to some armies as the most promising prophylactic drug, especially against soman (Anderson, Harris et al. 1992). (2) Another possibility is to administrate an enzyme that is capable of splitting an OP agent or (3) scavengers that are able to bind to an OP and therefore decrease the level of the poisonous agent in the organism. (4) And in addition to the mentioned possibilities, standard oximes used as antidotes exert prophylactic effects as well (Bajgar 2004).

### *(2) Medications*

There are three main classes of medication that are helpful in the management of intoxication: anticholinergics, oximes, and anticonvulsants.

**1.** Atropine is the classic anticholinergic drug, or more precisely antimuscarinic drug, recommended for the treatment of organophosphorus intoxications. It competitively and reversibly blocks the binding of ACh to the muscarinic receptor but has no significant effects at nicotinic receptors. This means that it is an effective treatment of parasympathetically mediated effects on excessive secretions and smooth muscle activation caused by nerve agents, but which can not treat muscle paralyis (Cannard 2006).

**2.** While atropine helps reverse the effects of parasympathetic overload, AChE reactivators such as one of the recommended pyridinium oximes (pralidoxime, trimedoxime, HI-6, obidoxime, methoxime) are used for the treatment of organophosphate poisoning in humans by restoring the AChE activity the function of the NMJ. However, oximes are only effective before aging. The rate of aging is different for each NA. The half-time of aging for soman is only 2-6 min (Sidell and Groff 1974), therefore they are theoretically useless in soman exposures. Since oximes reactivate AChE, one might expect a global reversal of all effects, but in practice, they have little effect on muscarinic symptoms and signs (Cannard 2006).

**3.** The likelihood of convulsions is directly related to the dose of exposure. Diazepam is currently the anticonvulsant of choice for the treatment of prolonged seizures or the status epilepticus. A reduction in mortality of NA-exposed experimental animals treated with diazepam has been demonstrated, particulary when used in combination with atropine (Cannard 2006).

## *E. Acetylcholinesterase Reactivators*

Although the phosporylated esteric site of AChE undergoes hydrolytic regeneration at a slow or negligible rate, Wilson (1951) found that nucleophilic agents, such as hydroxylamine ($NH_2OH$), hydroxamic acids (RCONH-OH) and oximes (RCH=NOH), reactivate the enzyme more rapidly than does the spontaneous hydrolysis. Selective reactivation is achieved by a site-directed nucleophile, where the interaction of a quaternary nitrogen with the negative subsite of the active center would place the nucleophile in close apposition to the phosphorus. Wilson and Ginsburg (1955) proved that with pyridine-2-aldoxime methyl chloride (pralidoxim) occurs the reactivation at a million times the rate of that with hydroxylamine (Goodman and Brunton 2008). The oxime moiety is oriented proximally to exert a nucleophilic attack on the phosphorus; a phosphoryloxime is formed, leaving the regenerated enzyme (Wilson 1959). Several bis-quaternary oximes were shown subsequently to be even more potent as reactivators for insecticide and nerve gas poisoning.

### 1. Pyridinium Oximes

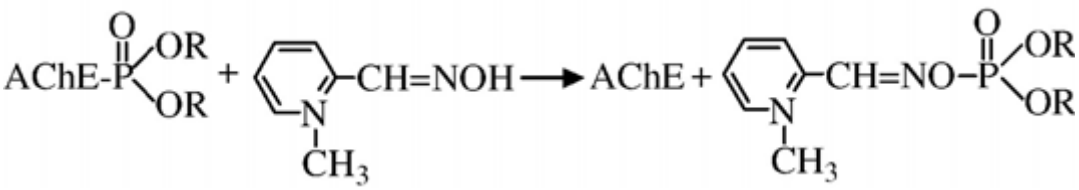



**Figure 6:** Reactivation of phosphorylated acetylcholinesterase with pralidoxime and formation of reactivated enzyme and phosphorylated oxime

Besides their reactivation potency, oximes bind to AChE as reversible inhibitors, form complexes with AChE (either in the active site or in the allosteric site or in both sites of the enzyme) and protect AChE from phosphorylation. When the reversible inhibitor binds to the active site, the protection is due to direct competition between the

organophosphorus compound and reversible inhibitor. Binding of a reversible inhibitor to the allosteric site induces indirect protection of the active site (Jokanovic and Stojiljkovic 2006). For oximes, the plasma concentration of 4 μl/ml is generally accepted as the minimum to counteract nerve poisoning (Kassa 2002).

## 2. Pyridinium oximes used in the treatment of OPC poisoning

We can divide them into two groups — the monopyridinium and bispyridinium oximes. The only currently used monopyridinium oxime is pralidoxime (2-PAM), while the most significant bispyridinium oximes comprise: trimedoxime (TMB-4), obidoxime (LüH-6, Toxogonin), HI-6 and HLö-7 (Dawson 1994). HI-6 is considered to be the most versatile reactivator, however its reactivation potency is not sufficient in the case of tabun poisoning. Here, obidoxime is the drug of choice as well as in the case of poisoning by pesticides (Jokanovic and Maksimovic 1995; Kassa 2002).

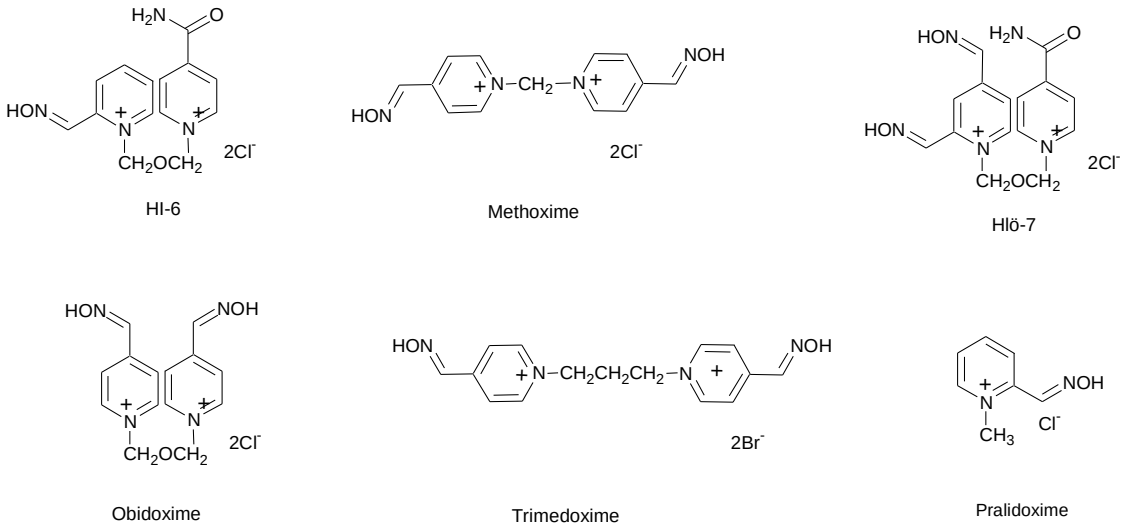
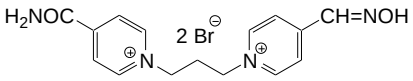
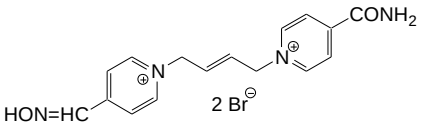



**Figure 7**: Commonly used oxime reactivators




K027




K203

**Figure 8:** New promissing compounds

## a) Pralidoxime (2-PAM)

Pralidoxime was synthesised in the USA in 1955 (Wilson and Ginsburg 1955) and is very efficient in reactivating of AChE inhibited with sarin or VX (Sidell and Groff 1974; Nozaki, Aikawa et al. 1995), but was not successful in reactivation of the tabun- or soman-inhibited enzyme (Koplovitz and Stewart 1994). 2-PAM, as quaternary pyridinium salt, does not easily penetrate the blood–brain barrier into the central nervous system.

## b) Trimedoxime (TMB-4)

It is the only of the major bispyridinium oximes with a propylene bridge between the two pyridinium rings. It was shown that TMB-4 is a more potent reactivator of the DFP-inhibited AChE than 2-PAM (Hobbiger and Sadler 1958) and better reactivator than LüH-6 in the case of the tabun-inhibited enzyme.

## c) Obidoxime (LüH-6, Toxogonin)

Obidoxim was synthesised in Germany and introduced into medical practice in 1964 . The new oxime showed immediately a significant potential as an antidote in poisonings with OPCs (Erdmann and Engelhard 1964). Similarly to 2-PAM and TMB-4, LüH-6 was also inefficient in soman poisoning in primates (Hamilton and Lundy 1989).

### d) HI-6

The first oxime that could reactivate soman-inhibited AChE and afford at least some protection of the animals experimentally poisoned with this nerve agent (Inns and Leadbeater 1983). The only drawback of HI-6 was that this oxime could not reactivate tabun-inhibited AChE (Clement 1982). But it still remains the most versatile antidote and therefore is used as the drug of choice.

### e) HLö-7

This latest important oxime reactivates AChE inhibited by any of the four major nerve agents (Worek, Kirchner et al. 1994) as well as the enzyme inhibited by cyclosarin (Lundy, Hansen et al. 1992).

### f) Newly developed oximes

K027 is a very good reactivator of tabun- and methylparaoxone-inhibited AChE in both *in vitro* and *in vivo* experiments with relatively low toxicity (Kuca, Bartosova et al. 2005; Petroianu, Nurulain et al. 2007). K203 is also promising compound of tabun-inhibited AChE (Kovarik, Calic et al. 2008) and in combination with atropine seems to be effective for a decrease in tabun-induced neurotoxicity (Kassa, Karasova et al. 2009).

## 3. Non-reactivation properties of reactivators

For a long time it has been thought that, besides performing AChE reactivation in organophosphate poisoning, oximes might also show some direct pharmacological effects. This was supported by observations that in some studies the antidotal effects of oximes could not be explained only on the basis of AChE reactivation (Clement 1981). In some experiments, only limited or no reactivation of soman-inhibited cholinesterase activity occurred when HI-6 was administered suggesting that some other mechanism of action of HI-6 other than cholinesterase reactivation is partly responsible for this

enhanced protective activity of HI-6 (Hamilton and Lundy 1989; van Helden, van der Wiel et al. 1992).

Many scientist wonder what kinds of mechanisms other than reactivation are involved in life-saving results after lethal intoxication by tabun and soman. Several models have been tested using different oximes and data have showed that the survival was due to a recovery of neuromuscular transmission in respiratory centers and in diaphragm (Hamilton and Lundy 1989; van Helden, van der Wiel et al. 1992). It was showed by the binding studies that there is a confirmed affinity of commonly used oximes to muscarinic receptors, similar to the nicotinic receptors. (Amitai, Kloog et al. 1980) Their inhibitory constants to the muscarinic receptors are in micromole range.

The mechanism of binding to the muscarinic receptors is still not fully unraveled, but allosteric manner is a possibility. The number of allosteric binding sites is still a matter of discussion even though it is suggested positive and negative allosteric modulators bind to the same or overlapping sites (Proska and Tucek 1995). It is possible that most of these modulators of muscarinic receptors bind to the same site on the receptor, since most of these modulators decelerate the association and dissociation of ligands at the orthosteric site probably by obstructing the access of the ligand. It is also possible that some ligands bind to both orthosteric and allosteric binding sites simultaneously. The muscarinic M2 receptor is assumed to be more sensitive to allosteric ligands than other muscarinic receptor subtypes (Christopoulos, Lanzafame et al. 1998). For instance, obidoxim is considered to be an allosteric modulator of muscarinic M2 receptor (Ellis and Seidenberg 1992) or an allosteric antagonist (Grossmuller, Antony et al. 2006).

The antagonistic activity of oximes could be of importance in the search for other modes of actions, which may be complementary to the reactivating effect. In order to characterize the anticholinergic of selected oximes, the atria of the heart were chosen for functional examinations. In the atria, acetylcholine decreases the heart frequency by acting on muscarinic M2 receptors (Caulfield and Birdsall 1998). Both *in vitro* and *in vivo* techniques were used for examination.

# III.Aim

The aim of the thesis was to investigate whether or not “classical” reactivators obidoxime and HI-6 and the newly synthesized compounds K027 and K203 affect muscarinic receptor mediated responses differently in the atria and to compare the effective doses.

# IV. Materials and Methods

## *A. Animals*

Male rats of the Sprague-Dawley strain with the weight of 250-400 g were used in the current thesis. The Ethical committee of Göteborg University approved the study.

## *B. Materials*

Dormitor, Ketalar, Heparin (all Apoteket AB, Sweden), Adrenaline: phenylephrine (Sigma, St. Louis USA), Methacholine: 2-acetyloxypropyl-trimethyl-azanium (Sigma, St. Louis USA), Obidoxime(1,3-bis(4-hydroxyiminomethylpyridium)-2-oxapropane dichloride), HI-6(1-(2-hydroxyamino-methylpyridinium)-3-(4-carbamoylpyridinium)-2-oxapropane dimethansulfonate), K027(1-(4-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium) propanedibromi-de), K203([(E)-1-(4-carbamoylpyridinium)-4-(4-hydroxyiminomethylpyridinium)-but-2-ene dibromide) were synthesized at the Department of Toxicology, Faculty of Military Health Science, University of Defence, Hradec Králové, Czech Republic.

## *C. Data Analysis*

The tension was measured by MP100WSW data acquisition system (Biopac Systems, Goleta, CA, US) recorded and analysed on computer using the ACQ knowledge software (version 3.7.2 Biopac). Calculations were performed using Microsoft Excel 2000 and statistics were made by GraphPad Prism (version 4.01, GraphPad Software Inc.).

All data values are expressed as mean±s.e.m. Statistical significance was determined by one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni multiple-comparison test. All statistical analyses were performed on raw data, but the graphs are presented in percentages. P-values of 0.05 or less were regarded as statistically significant.

## *D. In vitro studies*

The rats were killed with carbon dioxide with premedication of Dormitor (medetomidin hydrochlorid 0,5mg/kg I.P.) and the organs (heart atria) were cut off the animals and fixed into the organ baths. It is necessary to keep them dry in sufficient amount of Krebs solution. The tissues were attached via a thread to isometric force transducer consisting of two steel rods where one is fixed and the other is adjustable. Each organ bath is filled with 25 ml of Krebs bicarbonate solution containing: NaCl 118 mM, KCl 4.6 mM, $CaCl_2$ 1.25 mM, $KH_2PO_4$ 1.15 mM, $MgSO_4$ 1.15 mM, $NaHCO_3$ 25 mM, and glucose 5.5 mM, bubbled by gas mixture of 95% $O_2$ and 5% $CO_2$. The temperature was kept at 37 ºC by a thermostat and pH = 7.4.

Krebs solution was prepared from deionised water, NaCl 6.9g/l, KCl 0.34g/l, $KH_2PO_4$ 0.16g/l, $MgSO_4$ 0.14g/l, $NaHCO_3$ 2.10g/l, glucose 0.99g/l, the solution was bubbled by gas mixture of 95% $O_2$ and 5% $CO_2$ for at least 45 minutes before $CaCl_2$ 0.18g/l was added and then it was bubbled by the same gas throughout the experiment to keep the stable neutral pH. All drugs additions were performed using a volume of 125 µl.

### 1. Atrial preparation

The heart was isolated and an atrial strip was prepared. The atria were placed on a bipolar electrode consisting of 3 rods – first one was the strip holder and second two provided the electrical stimulation from the stimulator. Atria were at the same time fastened to the adjustable rod to record changes in tension. The tension was adjusted during the stabilizing period of 30 minutes to a level of 4-7 mN. After stabilization, the atria were stimulated at 1 Hz at supramaximal voltage (50 V), delivered as square wave pulses with duration of 0.8 ms (stimulator: STM 100C, Linton, Welwyn Garden City, UK). Later, the doses of adrenaline and methacholine both in volume of 125 µl to reach the concentration of $10^{-4}$ M in the bath were administered. Then the increasing doses of oximes in concentration of $10^{-7}$ to $10^{-4}$ were administered in 10 minutes intervals in order to determine their antimuscarinic qualities as acetylcholinesterase reactivators.

### 2. Protocol for atria

1. Start recording on the computer
2. Turn on the continuous stimulation (1 Hz)
3. Waiting until a stable recording is reached
4. Add 125 µl of adrenaline at appropriate concentration to get $10^{-4}$ M in the bath
5. Wait until it gets stabilized
6. Add 125 µl of metacholine at appropriate concentration to get $10^{-4}$ M
7. After stabilization add 125 µl of appropriate increasing concentration of oximes (K027, K203, HI-6, Obidoxime) to obtain $10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$ M in each bath
8. Switch the continuous stimulation off

## *E. In vivo studies*

The preparatory methods were generally described previously by Tobin. G *et al.* (Tobin and Sjogren 1995).

The rats were anaesthetized with Dormitor (35mg/kg I.P.) and Ketalar i.m. (50mg/kg I.M.). During the experiment the Dormitor was infused via the femoral vein for maintenance of anaesthesia, when necessary. The body temperature was kept at 37°C by means of a thermostatically controlled blanket connected to a thermister inserted into the rectum. The arterial blood pressure and heart rate were monitored continuously via a catheter inserted into the femoral artery. The catheter was filled with saline and small amount of heparine. Barometer used in the experiment was calibrated. For maintaining a free airway, a cannula was placed in the trachea of the rat after tracheotomy. The left vagal nerve was exposed in the neck and a bipolar electrode placed under the nerve. Sympathetic nerve fibres running in close contact with vagal nerve were identified and cut. To prevent afferent activation, the vagal nerve was cut coronally. The muscarinic activation was performed by electrical stimulation of the vagal nerve (10 Hz, 8 V square-wave; 2 msec pulse-width).

After reaching the stable basal blood pressure reading, an increasing dose of the oximes was administered through the femoral vein. The doses of the drug used were 10µg/kg, 100µg/kg, 1mg/kg, 10mg/kg respectively. The animals were killed with an overdose of dormitor at the end of the experiments. Recordings were recorded externally, using pressure transducer (Biopac Systems, Goleta, CA, US) and evaluated by the software ACQ Acknowledge (Biopac Systems, Goleta, CA, US).

# V. Results

In the anaesthetized rats, the basal heart frequency was 380±31 bpm (n=23) before application of any experimental procedure. At the onset of electrical stimulation of the vagal nerve at 10 Hz, the heart frequency dropped to 312±27 bpm (n=23; $p<0.01$). The decrease in the heart frequency was more pronounced by HI-6 and obidoxime in comparison with K203 and K027.

The reduction of heart frequency in response to the ongoing electrical stimulation was dose-dependently inhibited by the administration of any of the four oximes. The maximum inhibition of the frequency decrease cause by the vagal stimulation was at 100 µg/kg *i.v.* by HI-6 (n=5; -12±3%; $p<0.01$), K027 (n=5; 100±1% of basal frequency; $p<0.001$) and obidoxime, while the maximum inhibition by K203 occurred at 1 mg/kg *i.v.* (n=5; 110±4% of basal frequency; $p<0.001$) although the dose of 100 µg/kg *i.v* already caused inhibition with $p<0.001$ (n=5).

## *A. In vivo effects on heart frequency*

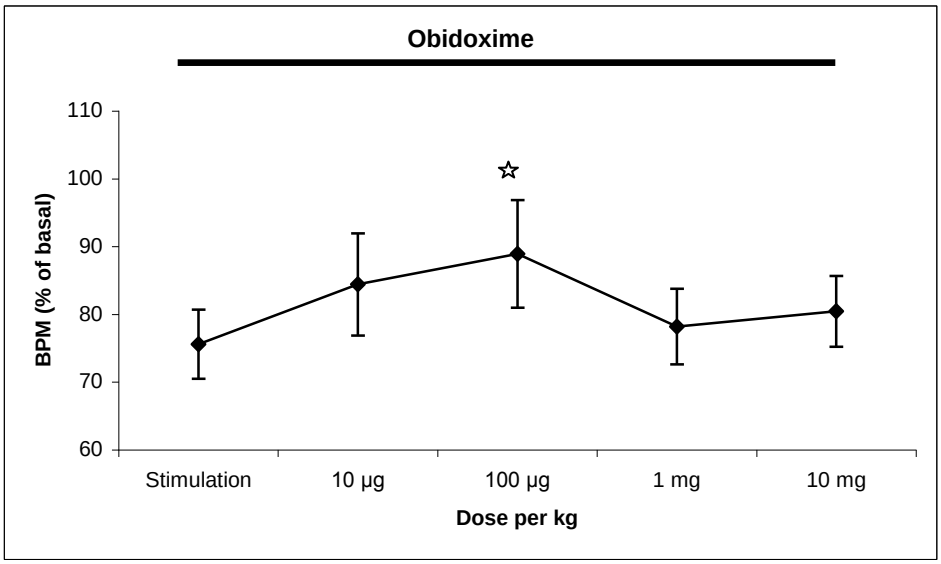



**Figure 9:** The effect of obidoxim on BPM in the anesthetized rat. Changes in heart frequency in anaesthetized rats in the absence and presence of reactivators during the vagal stimulation. Star indicates statistical significance: *($p<0.05$). Horizontal bar indicates the stimulation. All values are expressed as mean±s.e.m.

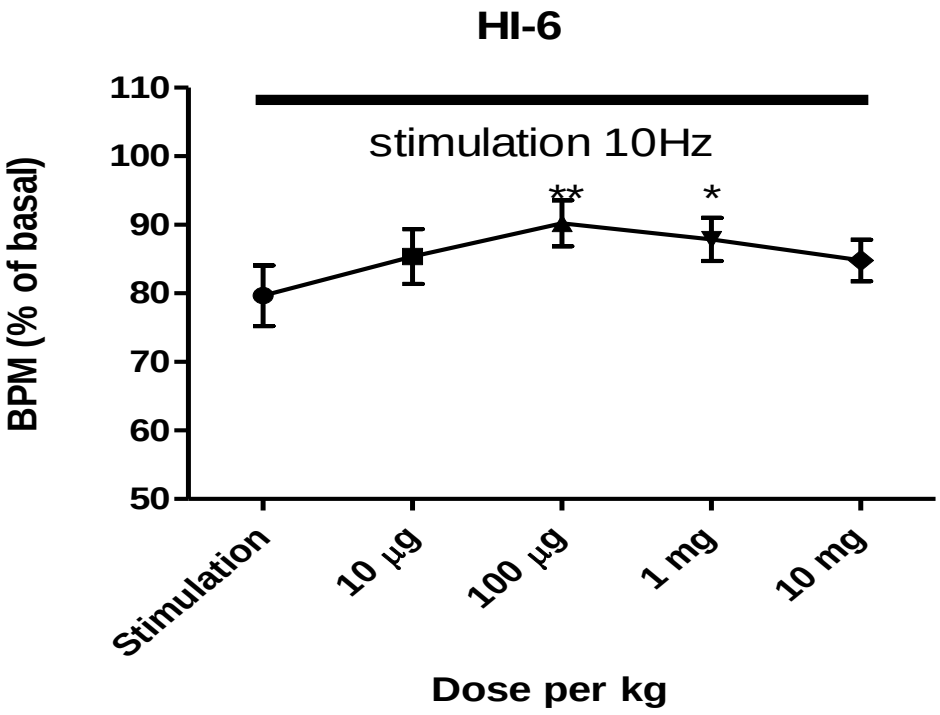



**Figure 10:** The effect of HI-6 on BPM in the anesthetized rat. Changes in heart frequency in anaesthetized rats in the absence and presence of reactivators during the vagal stimulation. Stars indicate statistical significance: *($p<0.05$) **($p<0.01$). Horizontal bar indicates the stimulation. All values are expressed as mean±s.e.m.

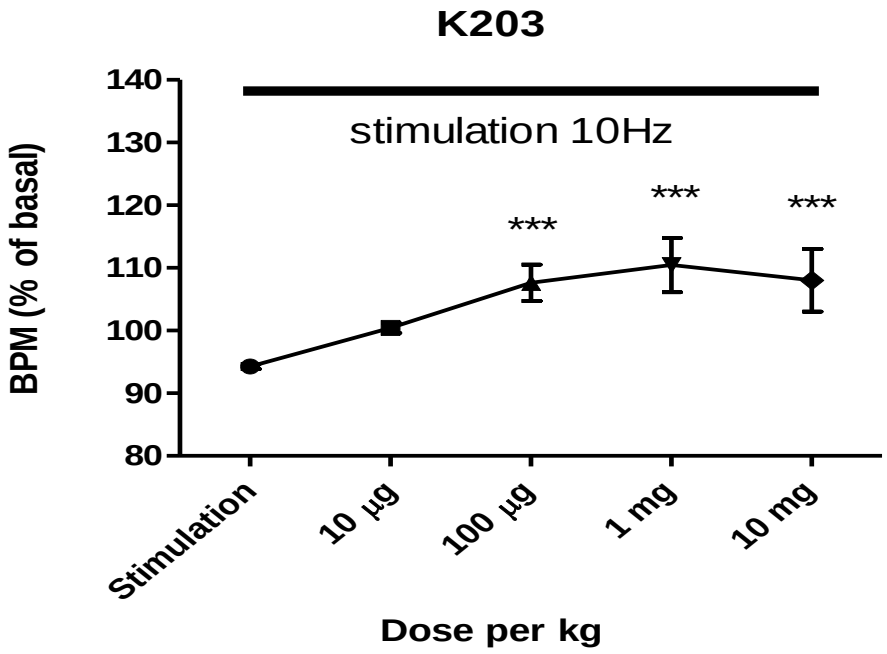



**Figure 11:** The effect of K203 on BPM in the anesthetized rat. Changes in heart frequency in anaesthetized rats in the absence and presence of reactivators during the vagal stimulation. Stars indicate statistical significance: ***($p<0.001$) Horizontal bar indicates the stimulation. All values are expressed as mean±s.e.m.

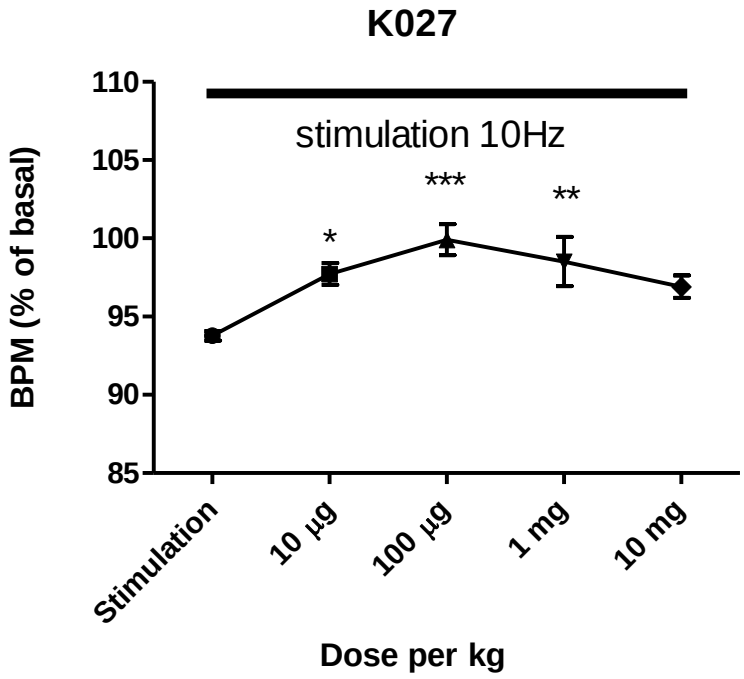



**Figure 12:** The effect of K027 on BPM in the anesthetized rat. Changes in heart frequency in anaesthetized rats in the absence and presence of reactivators during the vagal stimulation. Stars indicate statistical significance: *($p<0.05$) **($p<0.01$) ***($p<0.001$) Horizontal bar indicates the stimulation. All values are expressed as mean±s.e.m.

### *B. In vitro effects on atrial frequency*

The spontaneous (supported by a low electrical stimulation 0.1 Hz), basal frequency of isolated atrial preparations amounted to 492±37 (n=20) bpm. The experiments were performed in the presence of adrenaline ($10^{-4}$ M) to response of which all other responders are normalized. The administration of adrenaline at $10^{-4}$ M increased the frequency to 535±18 bpm (n=20; $p<0.05$). Methacholine at $10^{-4}$ M reduced the frequency in all groups ((-13±3% ($p<0.01$), -12±1% ($p<0.001$), -12±2% ($p<0.05$) -31±2% ($p<0.01$) in the HI-6, K203, K027 and obidoxime group, respectively (n=5 in each group)). The addition of the oximes causes a dose-dependent increases in the atrial frequency. While HI-6 eliminated the methacholine induced frequency decrease at $10^{-6}$ M, K203 and K027 caused the same elimination at $10^{-4}$ M unlike by obidoxime where the response at any dose never reached the frequency before the addition of methacholine.

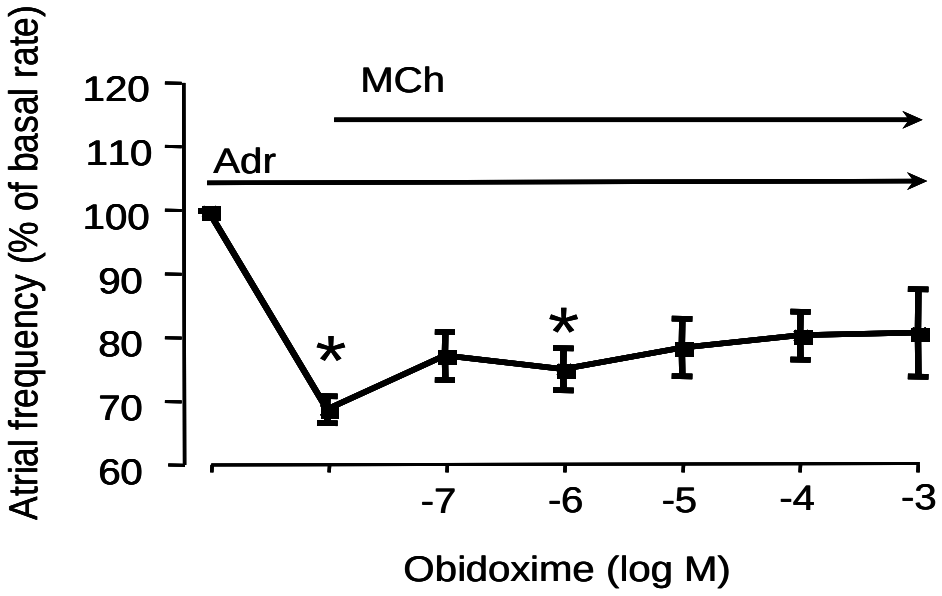



**Figure 13:** The effect of obidoxime on BPM in the isolated atria. Adr stands for adrenaline, MCh for methacholine. Horizontal arrows indicate the presence of drugs in the organ bath. Changes in frequency of isolated atrial preparations in the absence and presence of reactivators during chemical stimulation by methacholine ($10^{-4}$ M). All values are expressed as mean±s.e.m. The star indicates the statistical significance: *($p<0.05$).

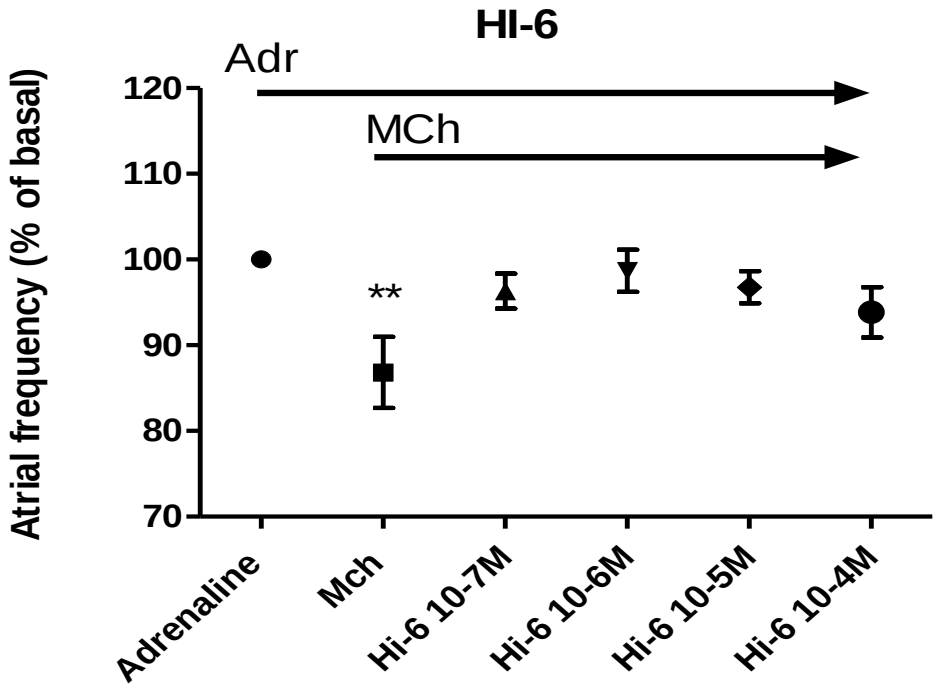



**Figure 14:** The effect of obidoxime on BPM in the isolated atria. Horizontal arrows indicate the presence of drugs in the organ bath. Changes in frequency of isolated atrial preparations in the absence and presence of reactivators during chemical stimulation by methacholine ($10^{-4}$ M). All values are expressed as mean±s.e.m. Stars indicate the statistical significance: **($p<0.01$).

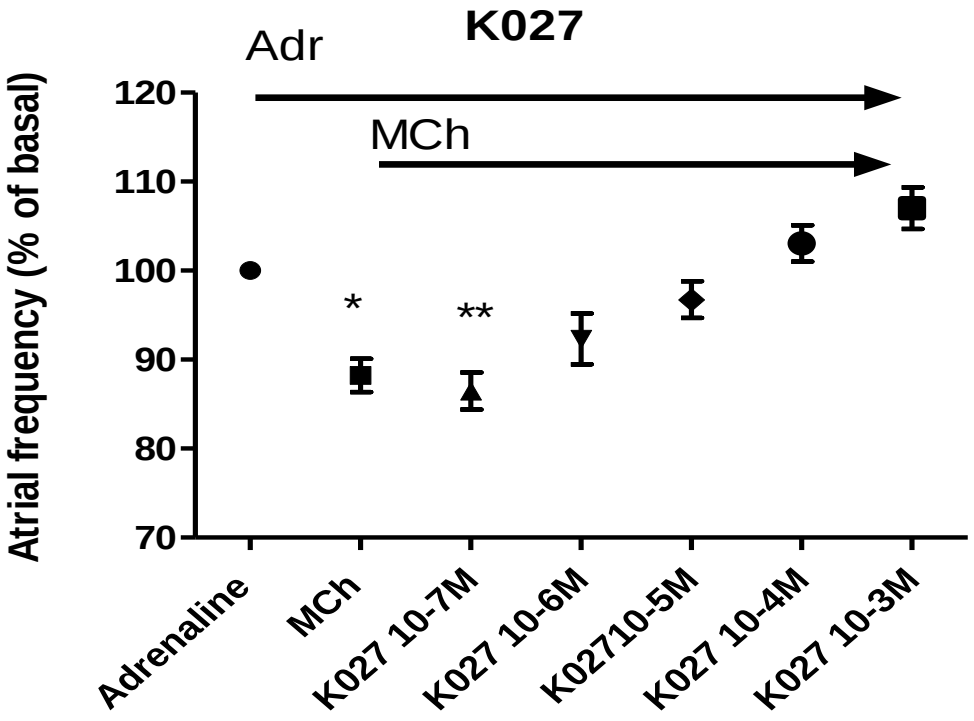



**Figure 15:** The effect of obidoxime on BPM in the isolated atria. Horizontal arrows indicate the presence of drugs in the organ bath. Changes in frequency of isolated atrial preparations in the absence and presence of reactivators during chemical stimulation by methacholine ($10^{-4}$ M). All values are expressed as mean±s.e.m. Stars indicate the statistical significance: *($p<0.05$) **($p<0.01$).

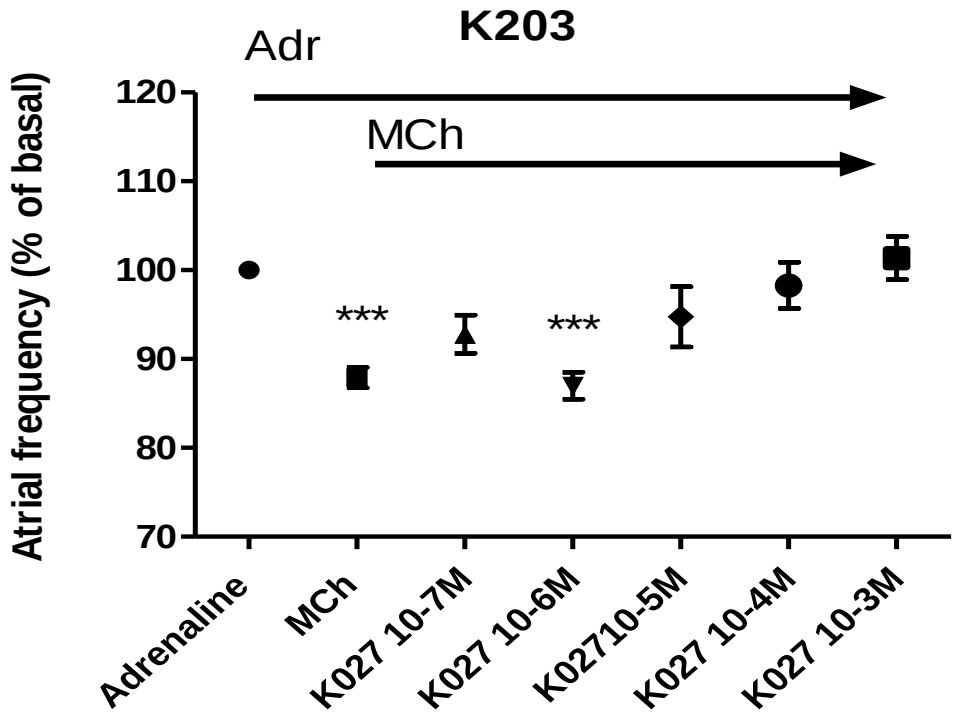



**Figure 16:** The effect of K203 on BPM in the isolated atria. Horizontal arrows indicate the presence of drugs in the organ bath. Changes in frequency of isolated atrial preparations in the absence and presence of reactivators during chemical stimulation by methacholine ($10^{-4}$ M). All values are expressed as mean±s.e.m. Stars indicate the statistical significance: ***($p<0.001$).

# VI. Discussion

Oximes are compounds designed to reactivate acetylcholinesterase, which becomes inactive due to the inhibition via phosphorylation or phosphonylation of serine hydroxyl group at its active site by organophosphorus compounds such as tabun, sarin, soman, cyclosarin or parathion (Goodman and Brunton 2008). Reactivating properties have already been tested and proved (Wilson 1959) several decades ago. Nowadays, further experiments are trying to unreveal non-reactivating properties of oximes (Tattersall 1993; van Helden, Busker et al. 1996). These include various effects of oximes such as actions on muscarinic ACh-receptors and effects on ion-channels associated with the nicotinic ACh-receptors (van Helden, Busker et al. 1996).

This study was designed to show the effect of AChE reactivators on the muscarinic receptors, i.e. non-reactivating property of oximes. Rat atria have been chosen as the model tissue, where M2 receptors occur so their influence on the heart rate could be examined. M2-receptor activation is responsible for the vagal inhibition of the heart rate (Rang and Dale 2007). The frequency has been examined by both *in vitro* and *in vivo* techniques.

During the *in vitro* experiments, the atrial preparations were exposed to a low frequency stimulation to provoke spontaneous activity. In order to enhance the possibility of cholinergic effects on the atrial frequency, adrenaline was added initially. Adrenaline is a non selective agonist of all adrenergic receptors and in the body basically acts against ACh, concretely increases heart rate (Goodman and Brunton 2008). Thereafter, methacholine, a muscarinic agonist, was administered as a reference of maximal receptor response. All compounds exerted an antimuscarinic effect, i.e. all oximes re-increased methacholine-decreased heart frequency. Response for adrenaline was taken as 100% and all other responces referred to this value. First concentration of K027, which reached the statistical significance was 1 μM and with every higher concentration increased even more up to proximately 110% of the basal frequency. That was the highest increase in frequency of all tested oximes. In the case of K203, the frequency was also slightly increasing above 100% - the maximum was at the 1 mM. It should be pointed out, that blocking effect of any compound probably should not cause

exceeding of 100%, so other mechanism like symphatetic stimulation may occurred, but more probably this was only due to variation of results and small number experiments. In case of K203 the first significantly effective dose was 10 μM, the significant effect at 0.1 μM might be only an artifact since we could expect some higher variation in the lower concentrations. HI-6 seems to be very effective, already at the lowest tested concentration (0.1 μM) with the maximum at 1 μM and then very slightly decreasing. This could be caused by the higher affinity HI-6 towards the enzyme and its inhibition with the higher concentration of oxime (Bajgar 2004). Although, in the study where inhibition constants were determined, HI-6 showed similar potency to inhibit the enzyme as K203 (Sinko, Brglez et al. 2010).

Also already the lowest tested dose of obidoxime, 0.1 μM, displayed its antimuscarinic effect (the significant effect at 1 μM can be again taken as an artifact). Although further increase in dose also increased the heart frequency but it hardly reached 80% of the response for adrenaline. In this respect, obidoxime could be regarded as a weaker muscarinic antagonist. However obidoxime has been reported as a weak muscarinic antagonist but usually stronger that other oxime reactivators (van Helden 1996).

The main aim of this study was to give the answer whether the oximes affect muscarinic receptors or not. The results comfirmed the hypothesis and this finding supports previous experiment with obidoxime where obidoxime has been reported as an allosteric modulator of muscarinic M2 receptor (Ellis and Seidenberg 1992).

The possibility of the release of acetylcholine from the cholinergic nerves by the oximes themselves was another question which was raised in relations with their non-reactivating effects. (Aas 1996) showed that neither obidoxime nor HI-6 but only Hlö-7 had a direct enhancing effect on the release of acetylcholine through activation or opening of $Ca^{2+}$ channels. Thus, we could expect that neither our *in vitro* experiments were influenced by this effect, at least not in the case of obidoxime and HI-6.

*In vivo* experimens, since vagal afferent stimulation reduces the heart rate in the anaesthetized rat (Saleh, Saleh et al. 2001) it was cut coronally to the point of stimulation which could lead to the elimination of afferent effects reduced variations in the results, probably by reducing the impact of compensatory mechanisms.

According to our observations, two contradictory effects could be observed. During the first phase, low dose of oximes caused an inhibition of the vagal effect on heart

frequency (vagal stimulation reduces the heart rate) by blocking muscarinic M2 receptors, which led to the increase in heart frequency. However, with the even more increased doses of oximes we could distinguish a phase, where the heart frequency decreased again. This phase was probably due to the inhibition of AChE. In other words, ACh, endogenously released by the vagal stimulation, could not be decomposed by the enzyme, was thus cumulated and caused the bradycardia. Comparable effect was described in the experiment, where oxime HGG-12 was tested in porcine cardiac atrial membranes (Reithmann, Berger et al. 1991). Although different method was used (competition binding experiment), the results show that the oxime is competetive antagonist without intrinsic activity at muscarinic receptors. The stimulatory action of HGG-12 on muscarinic acetylcholine receptors was, similar to our case, suggested to be due to partial inhibition of acetylcholinesterase by the oxime rather than to direct agonism at muscarinic acetylcholine receptors.

Nevertheless, this dual effect could not be observed in the *in vitro* experiments. This was probably due to the fact, that methacholine used as the cholinergic stimulator during the *in vitro* experiments was only poorly broken down by AChE and its actions are more prolonged (Martindale and Westcott 2008).

Comparing the individual compounds tested *in vivo*, muscarinic blocking properties can be reached already at the dose of 10 μg per kg only by K027, which is the lowest from all four oximes. Almost all compounds have the maximum effect on the muscarinic receptors at the dose of 100 micrograms per kg. The frequency decrease caused probably by the enzyme inhibition followed after this peak, however, it was not so pronounced by the oxime K203. This might be due to a lower affinity to AChE of this compound. This finding together with excellent reactivation potency in tabun poisoning and moderate toxicity (Kovarik, Vrdoljak et al. 2009) is making K203 a very promising antidote in organophosphorus poisoning especially caused by tabun.

# VII. Conclusion

All four tested oximes showed antimuscarinic properties. The results also showed that it is only matter of concentration and dose of the reactivator what makes the difference between muscarinic receptor antagonism and esterase inhibition. These findings supported the hypothesis that besides reactivation potency, oxime reactivators have some other, non-AChE-reactivating, effects that might be of importance in the treatment of organophosphorus poisoning. These effects, especially the anticholinergic properties, are still matter of discussion and further investigation is needed in order to characterize and quantify them.

# VIII. Závěr

Všechny čtyři testované oximy se prokázaly antimuskarinovými vlastnostmi. Výsledky také ukazují, že závisí pouze na koncentraci a dávce daného reaktivátoru, zda převáží antagonismus na muskarinovém receptoru, nebo dojde k inhibici esterázy. Tyto nálezy podporují hypotézu, podle které kromě reaktivace enzymu mají oximy nějaké další účinky, nezávislé na AChE reaktivaci. Ty by mohly být důležité v léčbě otrav organofosfáty. Tyto účinky, zvláště pak anticholinergní vlastnosti, jsou stále předmětem diskuze a je potřeba dalšího výzkumu, aby bylo možno je charakterizovat a kvantifikovat.

# IX. Abbreviations

ACh – acetylcholine

AChE – acetylcholinesterase

ATP - adenosine triphosphate

BuChE – butyrylcholinesterase

cAMP – cyclic adenosine monophosphate

CarbE - carboxyesterase

ChE – cholinoesterase

CN – nitril

CNS – central nervous system

DFP - diisopropyl fluorophosphate

LD50 - median lethal dose

mAChR – muscarinic receptors

NA – nerve agent

nAChR – nicotinic receptors

NANC – non-adrenergic non-cholinergic

NMJ – neuromuscular junction

NO – nitric oxide

OP – organophosphorus

OPC – organophosphorus compound

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