CHARLES UNIVERSITY

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Pharmacology and Toxicology

Evaluation of newly prepared insecticides *in vitro*

Diploma thesis

Supervisor: PharmDr. Marie Vopršalová, CSc.

Consultant supervisor: mjr. PharmDr. Vendula Hepnarová, Ph.D.

Hradec Králové 2020

Ondrej Tomáš

DECLARATION

I declare that this thesis is my original author work. All literature and other sources of information, that I used while processing, are listed in the bibliography section and they are properly cited.

Hradec Králové 2020

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ACKNOWLEDGEMENT

At first, I would like to express my gratitude to mjr. PharmDr. Vendula Hepnarová, Ph.D. for her professional leadership and for her help with writing this thesis. Sincere thanks to Assoc. PharmDr. Marie Vopršalová, CSc. For generous support and valuable comments.

Special thanks go to my family for their patience and support throughout my years of studies.

Abstract

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Title of diploma thesis: Evaluation of newly prepared insecticides *in vitro*

Malaria is a widespread infection and one of the most dangerous diseases transmitted by insects. It threatens lives of millions of people all around the world, thus its regulation is necessary. Most common malaria vectors are mosquitoes of genus *Anopheles.* Novel structures of insecticides with selective inhibition of mosquito acetylcholinesterase are subjects of research, with an intention to deal with this problem.

The aim of this work was to test six newly prepared succinimide derivatives with insecticidal potential. The ability of these compounds to inhibit *Anopheles gambiae* mosquito (*Ag*AChE) and human acetylcholinesterase (*h*AChE) was evaluated. Leading structures of these compounds were also tested to find relations between chemical structure and biological activity. The modified Ellman's method was used to obtain the half maximal inhibitory concentration (IC_{50}) values for both enzymes.

Tested substances were able to inhibit only *h*AChE and none of them displayed activity against *Ag*AChE. Compound LG-488 possessed IC_{50} value for human enzyme in the same range as tacrine. Even though these compounds are not suitable as insecticides, further modifications could lead to finding new structures able to successfully fight pest insects.

Key words: acetylcholinesterase, insecticide, malaria, Ellman´s method

Abstrakt

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Názov diplomovej práce: Hodnotenie novo pripravených insekticídov *in vitro*

Malária patrí medzi najnebezpečnejšie infekčné ochorenia prenášané hmyzom. Každoročne ohrozuje milióny životov po celom svete. Preto je nutné hľadať spôsoby ako zabrániť jej prenosu na ľudí. Jednou z metód je regulácia populácie jej najčastejších prenášačov, ktorými sú komáre z rodu *Anopheles*. Nové insekticídy schopné inhibovať komáriu acetylcholinesterázu by mohli byť nástrojom pre riešenie tohto problému.

Cieľom tejto diplomovej práce bola analýza šiestich novo pripravených derivátov sukcinimidu s inekticídnym potenciálom. U týchto štruktúr bola meraná schopnosť inhibovať enzým acetylcholinesterázu človeka (*h*AChE) a komára druhu *Anopheles gambiae* (*Ag*AChE). Štyri základné štruktúry týchto molekúl boli taktiež podrobené rovnakému meraniu s účelom nájsť vzťah medzi ich štruktúrou a biologickou aktivitou. Hodnoty polovičnej maximálnej inhibičnej koncentrácie (IC_{50}) pre oba enzýmy boli získané s využitím upravenej Ellmanovej metódy.

Testované látky boli schopné inhibovať *h*AChE, ale žiadna nevykázala výraznú aktivitu voči *Ag*AChE. Hodnoty IC_{50} pre ľudský enzým u látky LG-488 boli v približnom rozsahu ako u takrínu. Analyzované štruktúry sa síce neosvedčili ako insekticídy, avšak ďalšie modifikácie v štruktúre by mohli viesť k objaveniu látok, ktoré by predstavovali účinnú zbraň v boji proti škodcom.

Kľúčové slová: acetylcholinesteráza, insekticíd, malária, Ellmanova metóda

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A4	- asymmetric single AChE tetramer
A8	- asymmetric double AChE tetramer
A12	- asymmetric triple AChE tetramer
Aβ	- amyloid β-peptide
ACh	- acetylcholine
AChE	- acetylcholinesterase
AChE-E	- “erythrocytic” AChE
AChE-H	- “hydrophobic” AChE
AChEI	- acetylcholinesterase inhibitor
AChE-R	- “readthrough” AChE
AChE-T	- “tailed” AChE
ACT	- artemisinin-based combination treatment
AD	- Alzheimer’s disease
ADNB	- 5-(2-aminoethyl)-dithio-2-nitrobenzoate
*Ag*AChE	- mosquito acetylcholinesterase (*Anopheles gambiae*)
ALS	- amyotrophic lateral sclerosis
An.	- *Anopheles*
AO-AChE	- *Ace*-orthologous
AP-AChE	- *Ace*-paralogous
ARP	- acetylcholinesterase related (readthrough) peptide
ATChI	- acetylthiocholine iodide
BChE	- butyrylcholinesterase
BTChI	- butyrylthiocholine iodide
CA	- carbamates
ColQ	- collagen Q
CNS	- central nervous system
DDT	- dichlorodiphenyltrichloroethane
DMSO	- dimethyl sulfoxide
DTNB	- 5,5'-dithiobis-(2-nitrobenzoic acid)

G1	- globular AChE monomer
G119S	- glycine-to-serine mutation
G2	- globular AChE dimer
G4	- globular AChE tetramer
GABA	- γ-aminobutyric acid
GPI	- glycophosphatidylinositol
*h*AChE	- human acetylcholinesterase
IC_{50}	- half maximal inhibitory concentration
ITNs	- insecticide-treated nets
IRS	- indoor residual spraying
LLINs	- long-lasting insecticidal nets
LSM	- larval source management
NE	- neonicotinoids
NO	- nitric oxide
OC	- organochlorines
OP	- organophosphates
P.	- *Plasmodium*
PAS	- peripheral anionic site
PRiMA	- proline-rich membrane anchor
PY	- pyrethroids
RTS,S/AS01	- anti-malarial vaccine
SAM	- substrate-adhesive molecules
-SH	- thiol group
SI	- selectivity index
SEM	- standard error of mean
*Tc*AChE	- *Torpedo californica* AChE
THA	- tacrine
TNB^-	- 5-thio-2-nitrobenzoate
USD	- United States dollar
WHO	- World Health Organization
Yt	- Cartwright red blood cell antigen

LIST OF FIGURES AND TABLES

1. INTRODUCTION

Arthropods are responsible for a spectrum of health and agricultural problems worldwide (Engdahl 2017). They cause serious agricultural issues due to damage to the crops. It is estimated that around a quarter of annual worldwide crop output is destroyed by arthropods. This can lead to enormous financial losses (over 470 billion USD every year) and lack of food, which means a serious problem in developing countries (Culliney 2014). Besides that, it is inevitable to regulate their numbers to protect the human and animal population from infectious diseases (malaria, dengue, etc.) which they transfer (Engdahl 2017).

Malaria belongs to a group of the most spread and most common infectious diseases transmitted by insects. This infection is caused by protozoans of the genus *Plasmodium* (*P.*) (Daily 2017). It is estimated that in 2018, 228 million cases occurred, with the African continent as the most affected one with 93 % of all cases. During the same period, there were 405 000 deaths globally recorded, thereof 272 000 children under 5 years. Even though the number of cases is noticeably lower than in 2010 (251 million cases worldwide; 585 000 total deaths), it is still necessary to persist in the effort to fight this deadly disease (WHO 2019). This can be done by managing the populations of the most notorious malaria vector, mosquito *Anopheles gambiae* from genus *Anopheles* (*An.*). There are many more species (*An. arabiensis*, *An. funestus*, *Aedes aegypti*, etc.) able to efficiently transmit this infection (Sinka 2013). Not only they pose a threat to humans, but also the treatment of malaria is very expensive and, in many cases, ineffective. Thus, it is necessary to obtain the resources to tackle these organisms.

Humanity was searching for different ways to solve these issues for ages, from protection by natural repellents to physical barriers. Synthetic pesticides have proven to be more than an effective way to fight pests. Unfortunately, excessive contact with these substances has a negative impact on human health, beneficial animals, and the ecosystem as a whole (Oberemok et al. 2015, Grieneisen and Zhang 2018, Pennetier et al. 2008). World Health Organization recommends only four classes of insecticides of sufficient efficacy and safety for vector control (Najera and Zaim 2001). Moreover, frequent use of these substances increasingly leads to resistance of the insects, with the result of becoming completely ineffective. It is important to find the means that would promise

the least threat to non-target organisms, and represent an effective tool in the regulation of harmful insects at the same time (Oberemok et al. 2015, Grieneisen and Zhang 2018, Pennetier et al. 2008).

2. THEORY

2.1. Acetylcholinesterase

Acetylcholinesterase (AChE; E.C. 3.1.1.7) is a very important enzyme from the serine hydrolase family. This versatile protein can be found in many organisms around the world. AChE has high catalytic efficacy and is mostly associated with its presence in the synaptic cleft, where it helps to maintain proper neuronal signalization (Tripathi and Srivastava 2008, Kučera and Hrabovská 2013, Colovic et al. 2013).

2.1.1. Localization and function in human body

A large part of the nervous system consists of cholinergic neurons with acetylcholine (ACh) acting as a neurotransmitter. It provides signalization in the autonomic nervous system (all preganglionic neurons of sympathicus with addition of postganglionic neurons leading to sweat glands and whole parasympathetic innervation), preganglionic neurons to the adrenal medulla, all motoneurons, piloerector smooth muscles, numerous pathways in the brain and spinal cord. It is possible to say, that the cholinergic system plays a role in almost all vital functions. Thus, it is necessary to maintain its adequate activity (Colovic et al. 2013, Wehrwein et al. 2016, Aluigi et al. 2005).

Action potential in axolemma induces cascade that triggers the release of ACh from vesicles of axon terminal via exocytosis into the synaptic cleft. ACh molecules bind to receptors (nicotinic and muscarinic) placed on the postsynaptic membrane. This reversible interaction induces new action potential in the postsynaptic neuron. AChE accumulated in the postsynaptic membrane regulates this cholinergic transmission by hydrolysis of ACh into acetyl and choline (Figure 1.). This way it prevents excess stimulation of receptors by breaking down the redundant neurotransmitter in the synaptic cleft and helps to recreate resting potential (Figure 2.) (Tripathi and Srivastava 2008, Kučera and Hrabovká 2013, Colovic et al. 2013).

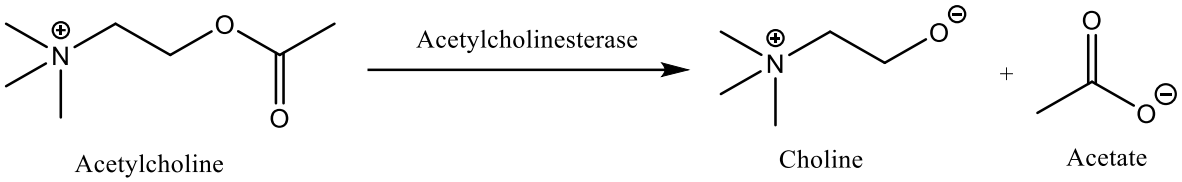


Figure 1. Hydrolysis of acetylcholine into choline and acetate.

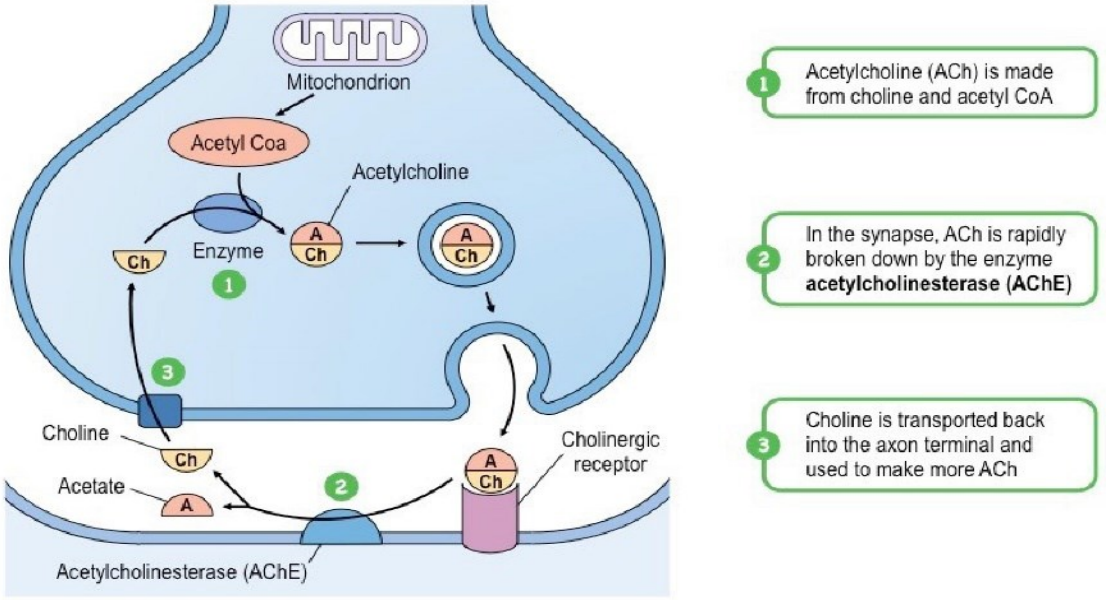


Figure 2. Cholinergic transmission.
Source: URL: http://opening.download/spring-opening.html. Obtained 10.3.2020

Accumulating evidence proves that AChE can also be found in non-neuronal tissues, where it has secondary functions in addition to its esterase activity in the nervous system. On the surface of erythrocytes, it carries Cartwright (Yt) blood group antigen (Colovic et al. 2013, Saldanha 2017, Offermanns and Rosenthal 2008), participates in the nitric oxide (NO) signalization, and anti-inflammatory process. Red blood cell AChE can be used for diagnosis of intoxication rates by pesticides and is taken into account as a biomarker of some disorders (ALS, glaucoma, etc.) (Saldanha 2017). The structure of AChE shows significant similarity to substrate-adhesive molecules (SAM) (Pickett et al. 2017). New findings point to AChEs ability of binding to laminin-1β, collagen IV, and amyloid β-peptide (Aβ) (Layer et al. 2005, Johnson and Moore 2004). This suggests that AChE takes part in cell polarization, organization, and communication (Pickett et al. 2017). Apparently, the expression of AChE in non-neuronal tissues is downregulated by butyrylcholinesterase (BChE) and engages in cell differentiation (Paraoanu et al. 2006,

Layer et al. 2005). It also plays a significant role in the development of nervous and hematopoietic cells (Johnson and Moore 1999, Taylor 1991), retina (Layer et al. 2005, Layer et al. 2013), skeleton (Pickett et al. 2017, Layer et al. 2013), kidneys (Layer et al. 2013), and gastrointestinal tract in embryogenesis of vertebrates (Pickett et al. 2017). Many of these functions are not connected to AChEs catalytic activity or synaptic signalization by ACh (Pickett et al. 2017, Landgraf et al. 2010). Unfortunately, most of this evidence is based on in-vitro, or animal in-vivo studies, so further research is needed to consider it relevant for human physiology.

2.1.2. Pathology

As already been mentioned, AChE belongs among structures with the purpose to maintain proper neuronal signalization. Due to abundant presence in the nervous system and potential function in development, it is obvious that improper function of this enzyme, or disbalances in its substrate ACh, can lead to severe outcomes. Multiple chemicals can interfere in AChEs activity intentionally (medicaments to treat distinct disorders) or unintentionally (chemicals used to regulate pests).

Intoxication by acetylcholinesterase inhibitors (AChEI) can induce life-threatening conditions. These substances were widely used as pesticides, but later was their use limited. They block the cleavage of acetylcholine by AChE, with following its cumulation in the synaptic cleft. This results in overstimulation of cholinergic receptors, which causes various adverse effects (muscarinic, nicotinic, and CNS symptoms), based on which pathways are affected. The severity of acute poisoning is dose-dependent. Serious intoxication is usually fatal because of cardiac or respiratory failure (Lionetto et al. 2013, Paudyal 2008).

Acute poisoning is not the only problem. Delayed polyneuropathy, chronic neurotoxicity, and behavioural impairment can occur weeks after intoxication (Paudyal 2008). Moreover, stress induced during intoxication leads to disbalance in the ratio of expressed AChE isoforms. The elevated quantity of the AChE-S variant makes the brain more susceptible to neurotoxicity and even doses below the threshold of inhibition can cause developmental defects. Also, a decrease in amounts of AChE-R isoform is present (Lionetto et al. 2013). AChE-R seems to represent the role of neuroprotector and reduces the formation of Aβ fibrils associated with Alzheimer's disease (AD) (Berson et al. 2008). Contact with organophosphates (OP) is not a condition for the development of AD and its

causes are still unknown. It is speculated that multiple factors are included in neurodegenerative progress.

Various diseases and disorders (AD, Parkinson's disease, glaucoma, Myasthenia gravis) are treated by blockade of AChE. Galantamine, rivastigmine and donepezil are reversible AChE inhibitors used as the treatment of choice for AD (Colovic et al. 2013, Offermanns and Rosenthal 2008, Santos et al. 2018). Some substances with cholinesterase inhibitory activity naturally occur in plants, thus they are subject of interest as potential medicaments (Santos et al. 2018).

2.1.3. Structure and forms

Primary structure of AChE monomer is twisted polymeric chain consisting of ~550 amino acid molecules (main thread of fixed amino acids, plus variable C-terminal peptide of 14, 26 or 40 residues and possible elongation by 60 or 66 residues) (Zimmerman and Soreq 2006) with molecular weight ~70 kDa. It is formed into 14 α-helices alternating with 12 almost completely parallel β-sheets (except one), thus AChE is classified as an α/β protein. Thanks to intermolecular interactions it takes on the shape of an ellipse with dimensions ~ 45 × 60 × 65 Å. Halfway through the enzyme leads a tight and deep gorge (Figure 3.) (Colovic et al. 2013, Dvir et al. 2010).

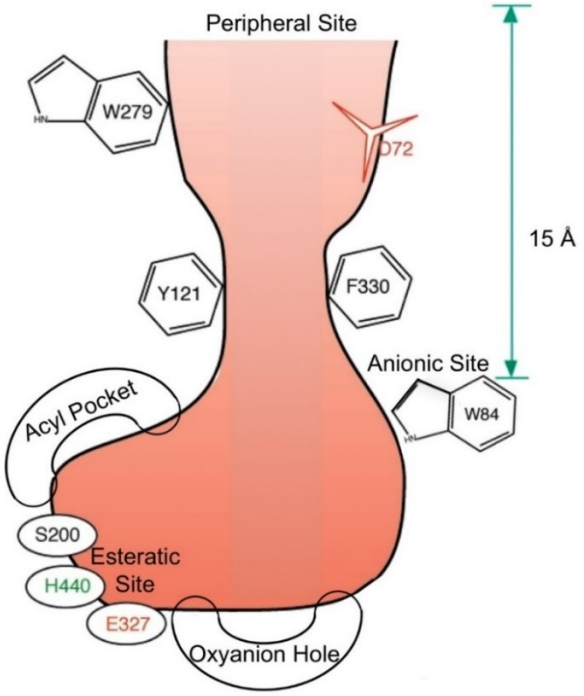


Figure 3. Acetylcholinesterase gorge with corresponding structures. Original figure of acetylcholinesterase of species Torpedo californica (TcAChE) that shares significant similarity to hAChE.

Source: Dvir et al. 2010

Gorge of AChE is roughly 20 Å deep and extends in diameter from its opening to the bottom. This cleft is the spot where the hydrolysis of substrates takes place because the catalytic active site (CAS) of AChE is situated here. The active site is located approximately 4 Å from the base of the enzymatic gorge. It contains two subsites – anionic and esteratic (Colovic et al. 2013, Dvir et al. 2010, Sussman and Silman 1992). A large part (>40 %) of the gorge surface represents a group of highly conserved 14 aromatic residues (Dvir et al. 2010) of tryptophan, tyrosine, and phenylalanine, extending to the anionic subsite (Ghatty Venkata Krishna et al. 2013). First, it was assumed that this part binds the quaternary nitrogen of Ach through electrostatic powers because of the presence of 6 to 9 negative charges in the cleft. Later this hypothesis was proven wrong when it was discovered that this site, which was earlier assumed to be anionic is, in fact, neutral and lipophilic in nature. Seems that acetylcholine is fixed by the interaction of choline part with π electrons of these aromatic residues. This is supported by the fact that Trp 86 is essential for catalytic activity, and its substitution by alanine can lead to a significant decrease in the efficiency of hydrolysis. It seems that also Phe 330 plays a significant role in the stabilization of the substrate along with Trp 86. Moreover, the line of these 14 aromatic residues is assumed to take part in the guidance of the substrate to the CAS.

The second subsite, esteratic, is placed on the bottom of the enzymatic gorge. It is responsible for the breakdown of the ester bond present in the molecule of substrate. The catalytic triad is located here, comprised of Ser 203, Glu 334, and His 440, all three lined up in a single plane. This amino acid triplet shares similarity with CAS of other serine hydrolases, which usually bear aspartate instead of glutamate (Colovic et al. 2013, Dvir et al. 2010, Sussman and Silman 1992, Cheung et al. 2013). Hydroxy group of serine creates tetrahedral intermediate with acyl part of a substrate while carbonyl oxygen accepts an electron (Dvir et al. 2010). Carboxyl ester is then hydrolysed resulting in the liberation of choline molecule and creation of temporary acyl-enzyme complex. The enzyme is regenerated through a nucleophilic attack by a water molecule, with help of His 447, releasing free acetic acid (Colovic et al. 2013). Scheme of the catalytic reaction is depicted in Figure 4.

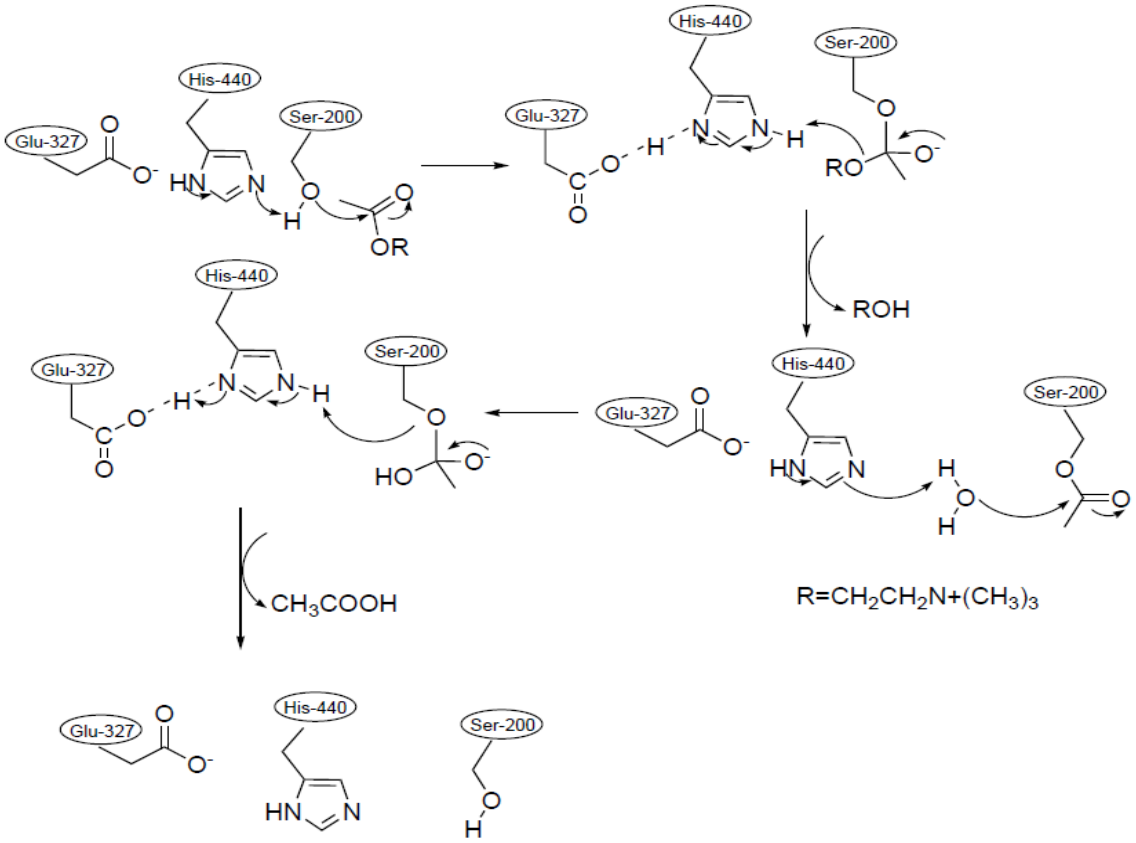


Figure 4. Mechanism of ACh hydrolysis catalysed by TcAChE.

Source: Colovic et al. 2013

In addition to the catalytic triad, esteratic subsite involves acyl pocket responsible for substrate specificity, and an oxyanion hole containing Gly 118, Gly 119, and Ala 201 (Dvir et al. 2010), which with other residues help to stabilize substrate in the correct positioning (Montella et al. 2012).

There are seven cysteine residues present in mammalian AChE. Six of them interact with each other through intra-molecular disulfide bridges resulting in the creation of omega loops. The seventh cysteine is located near the C-terminal end. This residue can link one catalytic subunit to another or to anchoring structures (Taylor 1991).

Besides choline-binding anionic subsite, there is at least one more peripheral anionic site (PAS) present around the gorge opening. This flexible domain comprises of six amino acids Tyr 72, Asp 74, Tyr 124, Glu 285, Trp 286, and Tyr 341 (Offermans and Rosenthal 2008), some of which are placed on omega loops. This domain plays a role in multiple AChE functions. Esteratic activity can be modulated through conformational changes in this region. Non-cholinergic properties are associated with PAS, such as linkage to laminin-1, collagen, and Aβ. Compounds able to interact with parts

of PAS can suppress neurite outgrowth and cell adhesion at various rates (Johnson and Moore 2004). Moreover, mutations in Asp 74 and Trp 286 notably reduce substrate inhibition, typical for AChE (Zimmerman and Soreq 2006).

AChE can take on several molecular forms, which differ in their structure, hydrodynamic properties, mode of attachment to the membrane surface, and distribution in tissues, while catalytic properties are preserved (Taylor 1991, Bon et al. 1979, Silman and Futerman 1987, Zimmerman and Soreq 2006).

Simpler forms can be used as intermediate products in the synthesis of more complex ones. Bon et al. (1979) divided them on the basis of quaternary structure into globular and asymmetric, marked as G and A respectively, while accessory digits represent the number of AChE subunits present in the structure. Globular species can sometimes exist as a monomer (G1) of a single enzymatic subunit (with approximately the same mass), but more common are dimers (G2) and tetramers (G4) created by the association of monomers (Bon et al. 1979) connected via disulfide bonds (Taylor 1991). G2 and G4 complexes are a lot heavier than the total sum of subunits, thus additional constituents seem to be present in their structures (Bon et al. 1979). G4 can associate with a proline-rich membrane anchor (PRiMA), which attaches them to the cell surface (Zimmerman and Soreq 2006). Asymmetric forms comprise of one (A4), two (A8), or three (A12) tetramers. Each tetramer is connected through a disulfide bond to one of three threads of the triple helical tail of Collagen Q (ColQ). The idea to call these forms asymmetric comes from the fact that this tail is extending in the opposite direction than multisubunit head (Taylor 1991, Bon et al. 1979, Silman and Futerman 1987). Alternative exons can be expressed to synthesize distinct parts of AChE (already mentioned three variants for C-terminus and two for elongation). Moreover, additional structural modifications are done posttranslatory (Zimmerman and Soreq 2006, Offermanns and Rosenthal 2008). Three basic isoforms occurring in humans are described in the following paragraphs and depicted in Figure 5.

AChE-T ("tailed" or "synaptic") is the most represented isoform in mammals and humans. Its main characteristic is 40 amino acids long C-terminal T peptide, bearing a cysteine residue in the third position from the end of the amino acid thread. This cysteine can link AChE-T subunits via disulfide bridges, resulting in the creation of homo-oligomers (G1, G2, G4) and hetero-oligomers. Tetramers can be attached to the cell

membranes trough already mentioned anchoring molecules ColQ (A4, A8, A12) or PRiMA (G4). PRiMA anchored G forms (preferred in brain synapses) are responsible for most of the catalytic activity (70-90%) in the central neural system (CNS), while asymmetric forms (preferred in neuromuscular junctions) mediate the rest (10-30 %).

AChE-R ("readthrough") is a single hydrophilic subunit. Its C-terminal peptide is called AChE related (readthrough) peptide (ARP), composed of 26 amino acids. In contrast to AChE-T, AChE-R does not have the ability to associate with other subunits nor anchor molecules. Thus, it is present only as a monomer. This isoform is stress-related and naturally AChE-R seems to play a role in multiple functions in human body (apoptosis) and health conditions (intoxication by AChE inhibitors, AD, etc.) (Kučera and Hrabovská 2013, Zimmerman and Soreq 2006, Offermanns and Rosenthal 2008, Hicks et al. 2011).

AChE-H ("hydrophobic") also known as AChE-E ("erythrocytic") or Yt is G2 dimers attached to the cell membrane through glycophosphatidylinositol (GPI) anchor. This isoform is predominantly occurring on the surface of erythrocytes or leucocytes (Kučera and Hrabovská 2013, Zimmerman and Soreq 2006, Offermanns and Rosenthal 2008).

The fourth isoform of the AChE subunit, called "snake" or "soluble" is completely absent in all mammals and humans. It occurs in some species of venomous snakes in form of a monomer, massively produced by venom glands, but can be found also in other tissues (Kučera and Hrabovská 2013, Frobert et al. 1997).

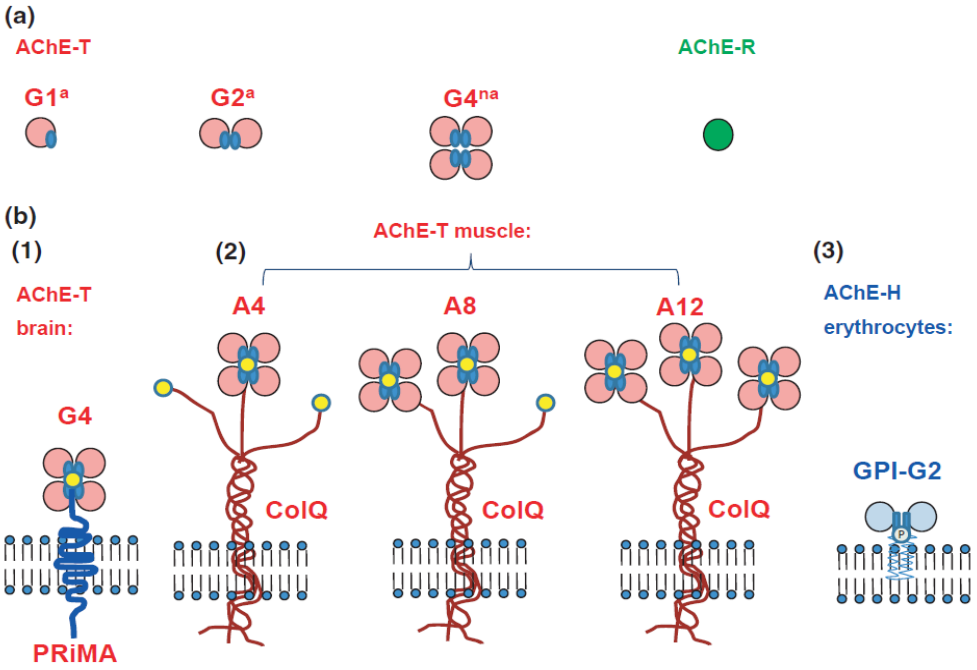


Figure 5. Summary of AChE isoforms present in the human body.

Source: Hicks et al. 2011

2.1.4. Differences between human and mosquito AChE

In its natural state membrane-anchored mosquito AChE shows no substrate inhibition characteristic for human protein. This feature disappears after the conversion of the insect enzyme into its non-amphiphilic form by thiocyanate (Dary and Wedding 1990).

The most markable difference between human (*h*AChE) and mosquito AChE is the number of genes that code this enzyme. In humans and mammals, just one AChE encoding gene is present, in contrast to two genes found in most of the insects (except some species of flies) (Pang et al. 2012). This duplication leads to the expression of two distinct AChEs, which were entitled by Kono and Tomita (2006) as *Ace*-orthologous (AO-AChE) and *Ace*-paralogous (AP-AChE) to prevent confusion (Kono and Tomita 2006).

Insect AChE contains additional cysteine residue near the CAS. This cysteine is replaced by phenylalanine in enzymes of fish, birds, mammals, and humans. In AP-AChE (responsible for most of the cholinergic functions) of *An. gambiae*, this Cys 286 residue (Figure 6.) is located at the gorge entrance (Pang et al. 2009, Dou et al. 2013). Moreover, Arg 339 is another residue unique to anophelines present on the edge of CAS (Pang et al. 2009). Targeting of these amino acids could be an effective way to tackle even insect species resistant to organophosphates, and have negligible or no effect on non-target

animals and humans, since these residues are missing in AChEs of most organisms (Pang et al. 2009).

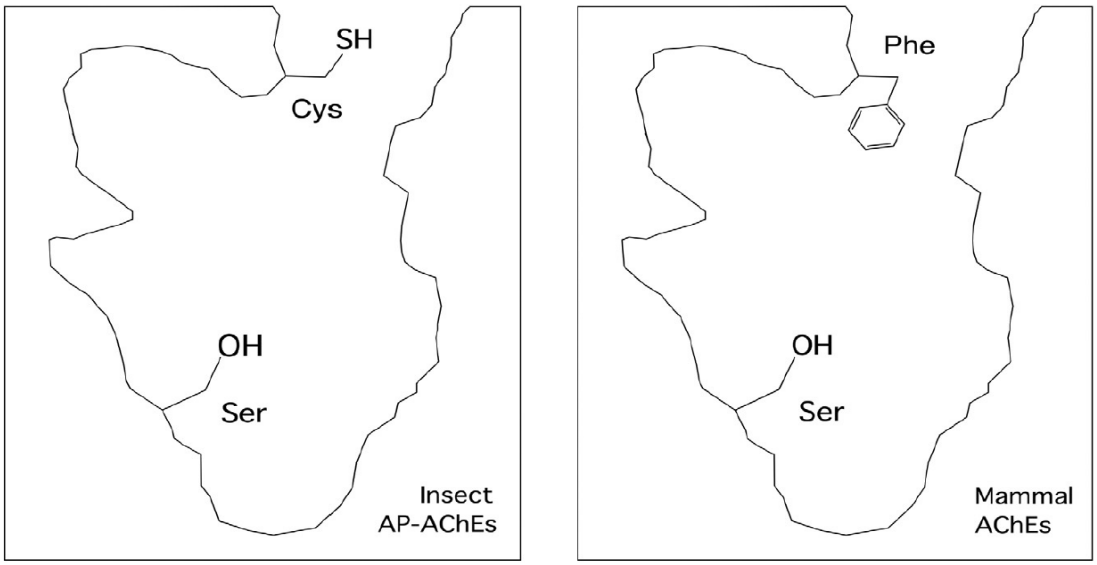


Figure 6. Structural difference at the peripheral site of the active-site gorge between insect (left) and mammal AChE (right).

Source: Pang 2014

Already mentioned resistance to AChE inhibitors is progressively more frequent every year. Due to the overuse of pesticides, multiple pests did undergo mutations that made them resistant to these compounds (Carlier et al. 2017). Substitution of Gly 119 by serine (G119S) in an oxyanion hole occurs in mosquito species *An. gambiae* and *Culex pipiens*, both able to transmit malaria (Kono and Tomita 2006, Carlier et al. 2017). Structures with the ability to tackle G119S resistant strains of mosquitoes are subject of interest in research along with compounds targeting insect-specific cysteine (Carlier et al. 2017) and arginine (Pang et al. 2009).

2.2. Butyrylcholinesterase

Butyrylcholinesterase (BChE, EC 3.1.1.8), also referred to as pseudocholinesterase or plasma cholinesterase is a protein with catalytic activity and belongs to the same enzyme family as AChE, but differs in location, substrate specificity, and structure. BChE is more abundant in blood plasma, but also present in the liver, brain, and lungs. The main function of this enzyme is still unknown, but its preference in plasma and liver points to the purpose of protection from xenobiotics and external substances (Offermanns and Rosenthal 2008).

BChE shares 58,3 % primary structure similarity with *Tc*AChE (Kučera and Hrabovská 2013). In contrast to AChE, amino acids Phe 288 and Phe 290 of the acyl-binding pocket are replaced by Leu 286 and Val 288 in BChE respectively. Another striking difference is that 6 of 14 aromatic residues found in AChE are aliphatic in BChE. These changes result in a more capacious enzymatic gorge and ability to bind larger substrates like butyrylcholine and cleave them faster than AChE does (Sussman and Silman 1992, Offermanns and Rosenthal 2008).

Just like in AChE, there is also diversity in BChE forms and their preference in tissues. The brain is rich in hydrophilic globular tetramers (G4), while asymmetric forms anchored to ColQ (A4, A8, A12) are present in neuromuscular junctions (Offermanns and Rosenthal 2008).

Besides esteratic activity, BChE has also some non-cholinergic functions. It takes part in cell proliferation and differentiation (Layer et al. 2005, Paraoanu et al. 2006), and pathological mechanisms (Layer et al. 2005). Moreover, expression of BChE is performed before the expression of AChE and down-regulates it (Layer et al. 2005, Paraoanu et al. 2006). BChE plays a significant role in organisms, thus it is important to carry on in its research.

2.3. Malaria

Malaria is an infectious disease caused by protozoan microorganisms from the genus *Plasmodium.* Five (*P. malariae, P. falciparum, P. vivax, P. ovale, P. knowleski)* of 172 known species in this genus can be successfully transmitted to humans and cause malaria (Talapko 2019). Most common in Africa is *P. falciparum*, responsible for the majority of cases (Engdahl 2017, Ashley et al. 2018). On Europe and Americas continents predominate *P. malariae* and *P. vivax*, Asia is also affected mostly by *P. vivax*.

These parasites are incorporated into the host's body through a bite of the infected female mosquito (Talapko et al. 2019, Ashley et al. 2018, Engdahl 2017). Several species can transmit malaria, but the most notorious vector is *Anopheles gambiae* of genus *Anopheles*. Also, *An. arabiensis*, *An. funestus* and *Aedes aegypti* are very efficient in the transmission of this disease (Sinka 2013).

According to estimation by WHO, in 2018, 228 million cases occurred worldwide. Africa as the most affected continent had 93% of all cases. During the same period, they recorded 405 000 deaths globally, thereof 272 000 children under 5 years. These statistics resemble a decrease contrary to 2010 when it was estimated at 585 000 total deaths and 251 million cases worldwide (WHO 2019). Nevertheless, it is necessary to keep looking for new and more efficient ways to deal with this problem.

The life cycle is rather complex consisting of two main phases (in mosquito and in human), each comprised of multiple stages. After the incorporation into the human body, *plasmodium* undergoes stages in the liver and then blood. In both, numbers of this pathogen are increased by repeating cycles of asexual replication. Part of parasites goes through development into male and female gametocytes, enabling sexual replication in mosquito's gastro-intestinal tract after transmission via blood meal. A special feature of *P. ovale* and *P. vivax* is the latent stage in the liver, which can induce relapses of malaria even years after the initial infection. Every species has a different primary incubation period (usually 10-21 days, depending on the species). After this interval, infection is demonstrated by symptoms, slightly diverse among strains. Non-complicated infection is manifested with non-specific signs, such as fever, diarrhoea, cough, chills, etc. Contrary to this, severe malaria has specific clinical features (respiratory distress, convulsions, prostration, disorientation, coma, etc.), but laboratory tests are the objective way of confirming the diagnosis (Ashley et al. 2018).

The first anti-malarial chemical was quinine (17th century) extracted from the bark of trees from genus *Cinchona* and even nowadays resembles an important drug to fight this disease. Unfortunately, quinine has a narrow therapeutic index (Achan et al. 2011). Since then, more drugs (mefloquine, chloroquine, amodiaquine, atovaquone, proguanil, etc.) came into use as well. Extraction of artemisinin from plant *Artemisia annua* led to the development of semisynthetic artemisinin derivatives (second half of the 20th century), such as artesunate (succinyl dihydroartemisinin) and artemether (dihydroartemisinin methyl ether), which provide well-tolerated, effective, and safe pharmacotherapy. Artemisinin-based combination treatment (ACT) is usually used for the management of uncomplicated malaria. ACT is a mixture of artemisinin compound with non-artemisinin based anti-malarial drugs. For now, parenteral artesunate is a drug of choice in the treatment of severe infection caused by *P. falciparum* in all patients including children and pregnant women. Possible alternatives are intravenous quinine,

parenteral quinidine, intramuscular artemether. Malaria is curable if diagnosed in time, but despite that, treatment is not always successful.

Sulfadoxine-pyrimethamine combination is used as chemoprophylaxis in children under 59 months and pregnant women. Atovaquone-proguanil, primaquine, mefloquine, or doxycycline are administered as prevention to travellers (Ashley et al. 2018).

First vaccine (RTS,S/AS01) in the four-dose regimen (booster after 18 months included) shown 36,3 % effectiveness against clinical malaria in children aged 5-17 months. WHO approved RTS,S/AS01 for pilot implementation in three countries (Malawi, Ghana, and Kenya) to gain further information about its properties. It targets sporozoite stages through surface protein, restricting the parasite to enter the liver cells. Vaccinations with different modes of action (targeting of merozoite stages, blockage of transmission) are the subject of interest in ongoing studies (Ashley et al. 2018, Laurens 2019).

Unfortunately, none of the anti-malarial medicaments can warrant full recovery with certainty. Moreover, parasites can develop resistance, which results in therapy failure. Minimalization of contact of humans and animals with malaria-transmitting insects decreases the risk of the disease to spread (Ashley et al. 2018). This is done through various methods of vector control (Ashley et al. 2018, Laurens 2019).

2.4. Vector control

Throughout history, humankind practiced various strategies to tackle pests. Substances able to kill insects (insecticides) of either inorganic (sulphur, lead arsenate, cryolite) or plant origin were already used in ancient times. Later, with an effort to create synthetic compounds, more efficient insecticides such as dichlorodiphenyltrichloroethane (DDT) were introduced (Oberemok et al. 2015). Unfortunately, pests can develop physiological (ability to withstand lethal doses of insecticide) or behavioural resistance (vector avoids places where insecticide is present). The action of dispersed chemicals slowly fades resulting in the decrease of their effectivity. The residual effect of insecticides indoors is usually 3-6 months, but can also last from week to one year, depending on circumstances (surface material, climate). To ensure efficiency, chemicals are repeatedly applied and classes with different a mode of action are rotated. Moreover, these chemical interventions proved themselves harmful to the environment and humans

(Najera and Zaim 2001). Thus, other methods to protect humans from mosquitoes are in use or are under research.

In anophelines both genders feed on plant nectar, but only females do bite, because the nutrients needed for egg production are present in the blood-meal. This means that males do not usually enter households and their exposure to insecticides is not so intense as in females (Engdahl 2017, Najera and Zaim 2001).

Some strategies are focused on both genders in the early stages of development in water bodies such as larval source management (LSM). Main goal is the reduction of numbers of adults able to transmit diseases by preventing larvae and pupae to reach adulthood. Among LSM interventions belong manual modification (drainage of surface water, coverage of containers by lids, etc.) and manipulation (water-level control, drain clearance, etc.) of reproduction habitats near the inhabited areas, the introduction of natural predators (larvivorous fish of the genus *Gambusia*, *Cyprinodontidae* family, etc.), and larviciding (repetitive treatment of water sources by chemical or biological insecticides; mineral oils as surface films, synthetic organic chemicals, insect-growth regulators, and historically Paris Green) (Walshe et al. 2017, Engdahl 2017, Tusting et al. 2013). But the effectiveness of LSM remains questionable. Studies which tested regulation methods based on the incorporation of larvivorous species of fish were mostly focused on the impact on numbers in the immature mosquito population, not the impact on adults or incidence of malaria in humans. Moreover, the results were incoherent and whereas this method seems to be nature friendly, new species can negatively affect native aquatic animals (Walshe et al. 2017). Contrary to this, habitat manipulation, habitat modification, and larviciding (alone or in combinations) in multiple cases showed significant reduction in vector prevalence (60-90 %) and malaria incidence (~75 %) (Tusting et al. 2013). Another biological approach could be the genetical modification of vectors (Huang et al. 2017, Engdahl 2017).

Many species of pests prefer to feed on animals (zoophilic) outdoors. That is why protective clothes are used during activities performed outside. Indoor residual spraying (IRS) is a method of application of insecticides on all possible surfaces in the building. Insecticide-treated nets (ITNs), also referred to as long-lasting insecticidal nets (LLINs), are bed-nets impregnated with insecticides, thus they resemble a combination of mechanical barrier and chemical protection. They are preferred during sleep against

vectors that usually feed on humans (anthropophilic) indoors, especially at night. Along with IRS, ITNs are a highly effective way to reduce human-vector contact in households (Walshe et al. 2017, Engdahl et al. 2016, Engdahl 2017).

Insecticides can be quite toxic to non-target organisms. Because of this, WHO recommended only four classes (pyrethroids, organochlorines, organophosphates, and carbamates) for IRS, from which only pyrethroids are approved for ITNs (Najera and Zaim 2001, Engdahl et al. 2016, Engdahl 2017). There is an urgent need for safe and effective novel compounds, which could provide reliable vector-control. Neonicotinoids are relatively new insecticides and their use has increased in last two decades (Oberemok et al. 2015).

2.4.1. Pyrethroids

Pyrethroids (PY) are synthetic derivatives of plant insecticides pyrethrins (Silver et al. 2014). The first generation was introduced in 1949 and became popular thanks to their low toxicity to non-target species. Relatively rapid degradation, when exposed to ultraviolet light, makes their application more difficult, but on the other hand, it prevents their accumulation in the environment (Oberemok et al. 2015). For now, only PY are recommended for the impregnation of ITNs (Najera and Zaim 2001, Engdahl et al. 2016, Engdahl 2017).

Pyrethroids prevent voltage-gated sodium channels placed in the postsynaptic membranes from closing. Prolonged opening causes the excessive flow of sodium ions making axon membrane unable to repolarize. This leads to paralysis of the organism.

This family consists of two types that differ in their structure. Permethrin, bioallethrin, and bifenthrin belong among type-I. Cypermethrin, deltamethrin, and fenvalerate are type-II pyrethroids that possess α-cyano group (Silver et al. 2014).

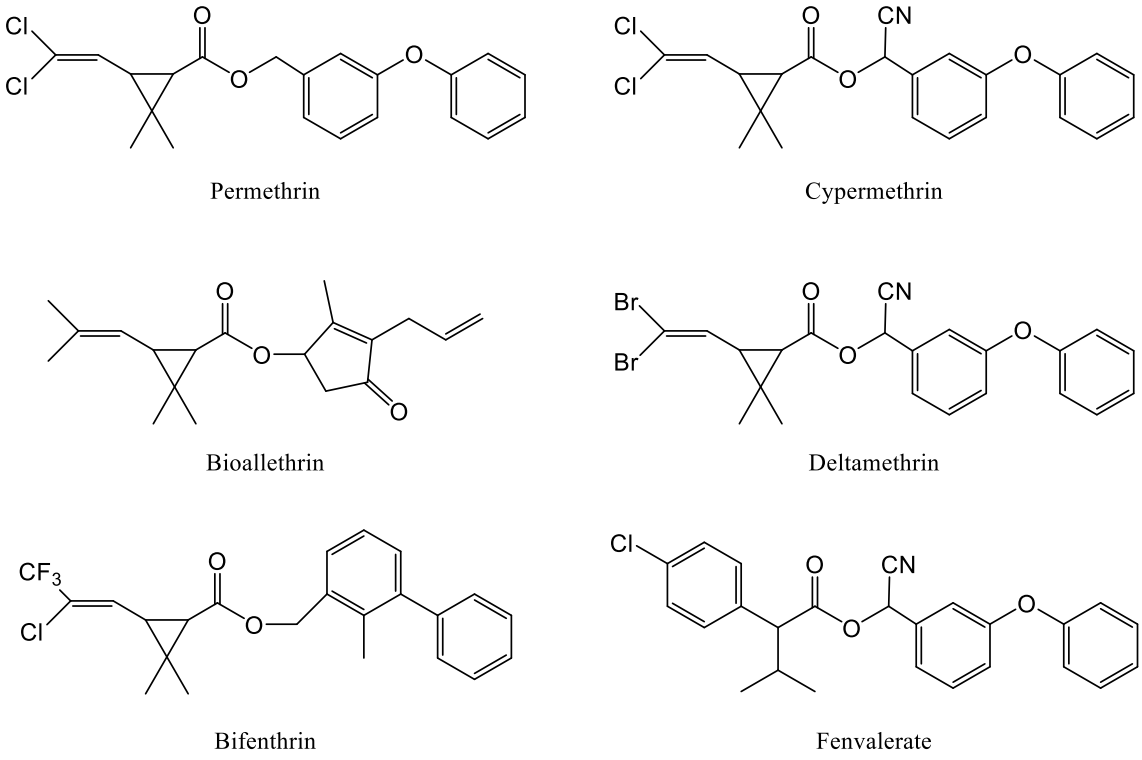


Figure 7. Structural formulas of pyrethroid insecticides.

2.4.2. Organochlorines

Organochlorines (OC) are highly persistent, chlorine-containing substances (Jayaraj et al. 2016). First organochlorine, DDT, was synthesized in 1874 and its insecticidal potential was discovered in 1939. Their use decreased after the introduction of OP and carbamates (CA) (Oberemok et al. 2015).

DDT like substances have a similar mode of action as pyrethroids, causing prolongation of sodium channels opening, paralysis, and death (Silver et al. 2014). Chlorinated cyclodines such as lindane are antagonists of γ-aminobutyric acid (GABA) receptors, thus restricting chloride ions to enter neurons. Chloride channels have an inhibiting effect on neurotransmission. Their protracted blockage leads to overstimulation and excessive release of neurotransmitters.

Some representatives of this group such as DDT, aldrin, dieldrin, and lindane (also known as γ-hexachlorocyclohexane) are still widely used in agriculture (Jayaraj et al. 2016) despite predisposition of resistance development in target pests and toxicity to the environment (Najera and Zaim 2001). Their structures are listed in Figure 8.

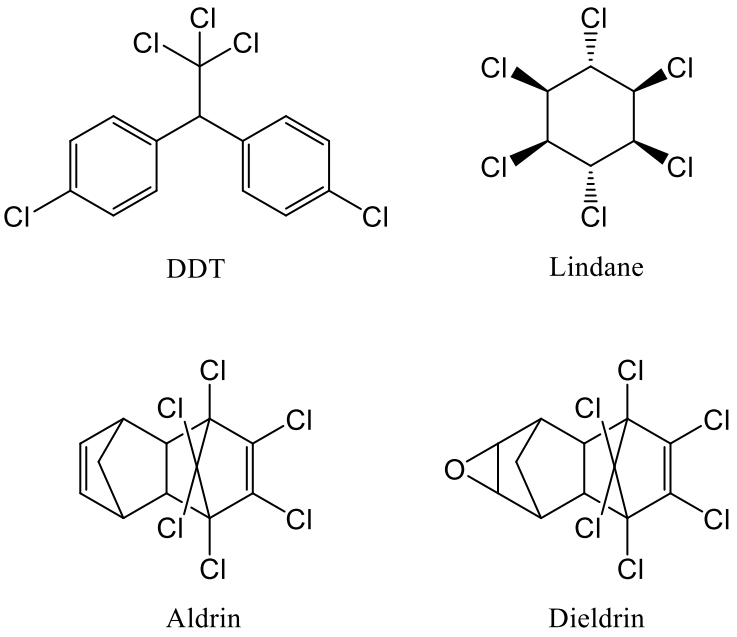


Figure 8. Structural formulas of organochlorine insecticides.

2.4.3. Organophosphates

First organophosphates were developed before the World War II with the intention to protect crops, but some of them (soman, sarin, and tabun) were abused in warfare as nerve gases. After that, their use was once again renewed in agriculture as insecticides but are still responsible for thousands of accidental deaths worldwide.

Structurally, OP compounds are considered as esters or anhydride derivatives of acids containing phosphorus. From over 100 compounds from this family, the most common are parathion, diazinon, chlorpyrifos, dichlorvos, malathion, and fenthion. Their structures are listed in Figure 9.

OP phosphorylate serine residue inside of the AChE active site. This irreversible inhibition leads to the accumulation of ACh in synaptic clef, resulting in overstimulation of all cholinergic receptors in the body. After a while, the enzyme-OP complex cannot be reactivated due to the process of ageing, and the only option to restore cholinergic activity is the synthesis of completely new proteins.

Acute poisoning is manifested by various symptoms according to which neurons are activated. These symptoms can muscarinic (miosis, elevated secretion of glands, emesis, cramps, loss of sphincter control), nicotinic (spams of skeletal muscles, paralysis, tachycardia, and hypertension), and CNS signs (confusion, tremor, convulsion, coma, etc.).

Depending on the dose and features of OP, intoxication by these compounds can be lethal. The main causes of death by OP are hypotension and respiratory failure. Fast initiation of treatment by atropine or oximes is vital. Supportive care consists of gastric lavage, administration of activated charcoal, or oxygen therapy (Paudyal 2008).

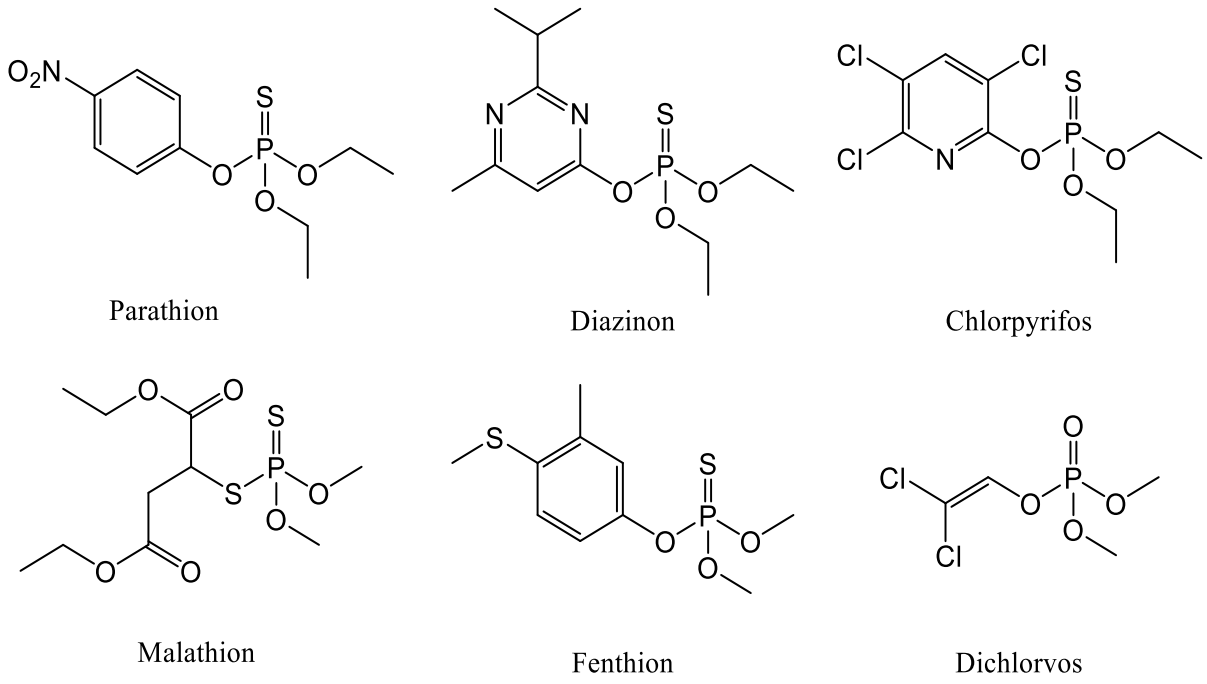


Figure 9. Structural formulas of organophosphorus insecticides.

2.4.4. Carbamates

Carbamates are esters of N-methyl carbamic acid introduced in the second half of the 20th century. In addition to their utilization in agriculture, they have been also used for the treatment of myasthenia gravis (Dias et al. 2015).

Representatives of this group are bendiocarb, propoxur (Najera and Zaim 2001), aldicarb, carbaryl, and carbofuran (Oberemok et al. 2015). Their structures are listed in the Figure 10.

Mode of action is similar to OP chemicals (AChE inhibition), but the complex with enzyme is rapidly reversible. Poisoning is not so severe as in OP and it can be treated by atropine and diazepam, but oximes are contraindicated (Najera and Zaim 2001).

Despite their lower toxicity, CA are not completely safe. They can induce various adverse effects (hepatic, renal) in non-target organisms and some of them possess endocrine-disrupting features (Dias et al. 2015).

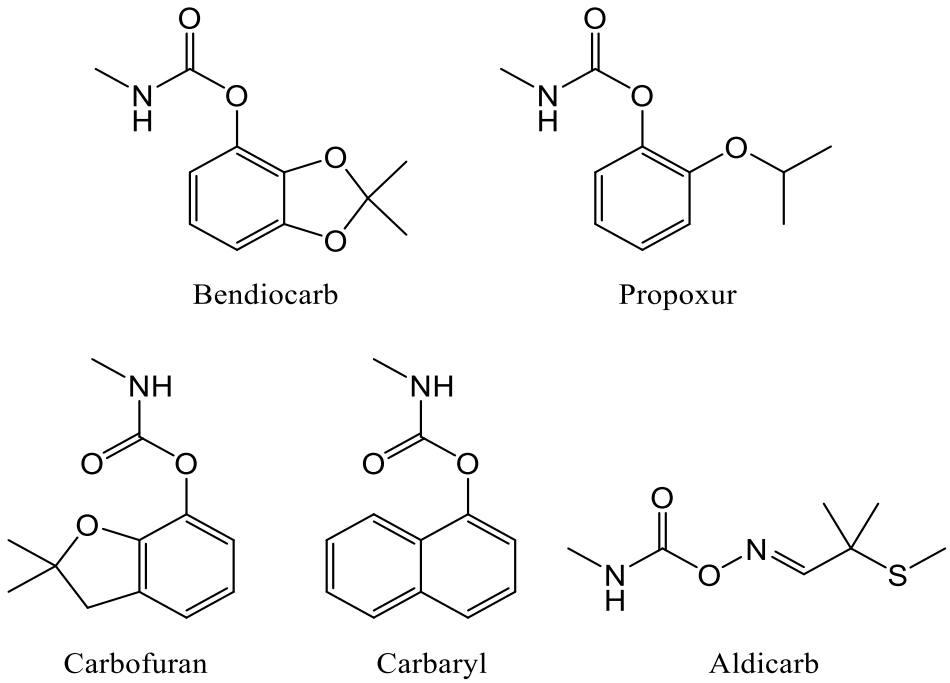


Figure 10. Structural formulas of carbamate insecticides.

2.4.5. Neonicotinoids

Neonicotinoids (NE) are relatively new synthetic compounds with a structure similar to nicotine. They became popular thanks to their lower toxicity to mammals in comparison to insects, but in recent years an opinion that they are potentially hazardous to bees and humans led to some restrictions in their use (Buszewski et al. 2019).

Imidacloprid was the first NE used on a large scale at the end of the 1990s. Later thiamethoxam and clothianidin were introduced (Buszewski et al. 2019), followed by thiacloprid, acetamiprid, and dinotefuran (Oberemok et al. 2015). Their structures are listed in Figure 11.

NEs are absorbed into plant tissues, providing systemic protection against pests (Buszewski et al. 2019). They are irreversible agonists of nicotinic acetylcholine receptors, causing paralysis after the intoxication of the target, due to excessive stimulation (Oberemok et al. 2015).

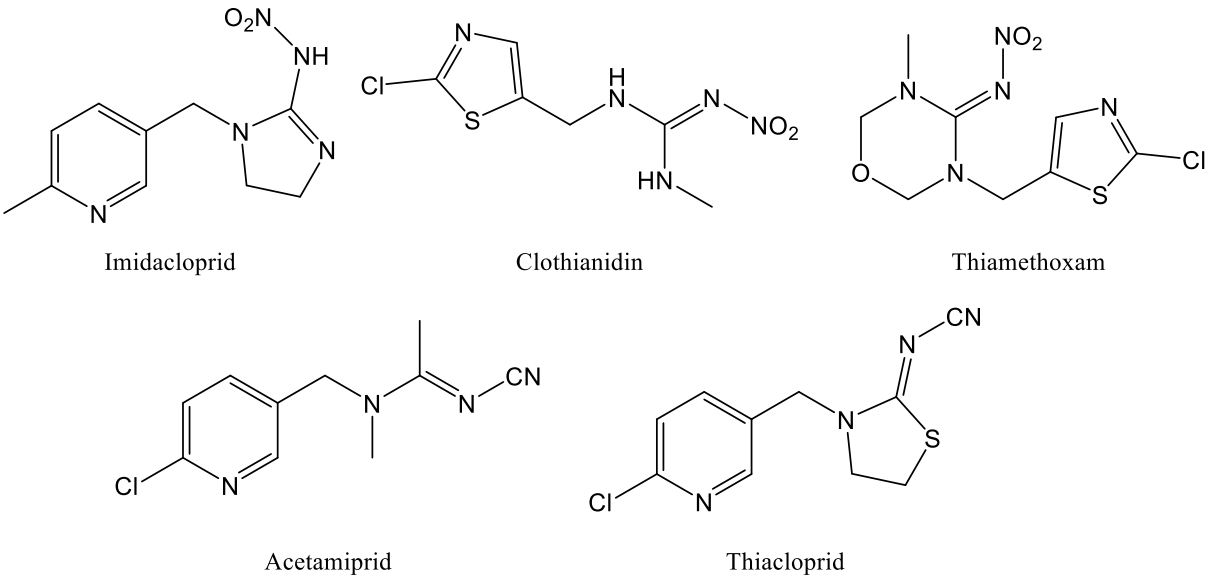


Figure 11. Structural formulas of neonicotinoid insecticides.

3. OBJECTIVES

The main objective of this thesis was *in vitro* determination of half maximal inhibitory concentration (IC_{50}) in succinimide derivates as potential inhibitors of acetylcholinesterase (AChEI) using modified Ellman's method. The selectivity and inhibition rate by six analysed structures was measured in human and mosquito acetylcholinesterase, and in butyrylcholinesterase. The best scenario would be the complete selectivity to mosquito enzyme, and none or at least negligible affinity to human AChE. An additional goal of this paper was an effort to reveal the relations between the structure and effect of these potential cholinergic inhibitors and their fragments.

4. EXPERIMENTAL PART

4.1. Ellman's assay

Modified Ellman's method was used for determination of catalytic activity of acetylcholinesterase and butyrylcholinesterase. Mechanism of this colorimetric method is based on the hydrolysis of acetylthiocholine ester by enzyme, creating the products of this process are thiocholine and acetate (Figure 12.). Due to thiol group (-SH) in its structure, thiocholine can be detected with help of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), also known as Ellman's reagent. Reaction of these two compounds (Figure 13.) result into production of yellow 5-thio-2-nitrobenzoate (TNB^-), capable of shift between two resonance forms (Figure 14.). Subsequently TNB^- is quantified spectrophotometrically by measurement of absorbance at 412 nm wavelength.

This method became so popular for its low cost, swiftness and simplicity and is widely used for monitoring intoxication in people, who are in contact with cholinergic inhibitors. However, in assay of blood samples, haemoglobin interferes due to absorption of visible light at the same wavelength as TNB^- does. Fortunately, this undesirable effect can be easily solved by diluting the sample, using double beam spectrophotometer, or by selection of different wavelength (Ellman et al 1961, Žďárová-Karasová et al. 2010).

In addition to cholinesterases, DNTB can be also used for assay of other enzymes such as arginase and peptide deformylase (Zhu et al 2004). Method can be modified by using different chromogenic substrates, usually acetylthiocholine iodide (ATChI) for AChE and butyrylthiocholine iodide (BTChI) for BChE (Žďárová-Karasová et al. 2010).

Other compounds are tested as substrates in an effort to maximize effectiveness and accuracy, and to minimize issues of this assay. For example, indoxylacetate does not react directly with oxime reactivators and thiols as DTNB does. The main problem is low water solubility of indoxylacetate and the time of assay has to be extended due to his 10 times lower reaction velocity (Pohanka et al. 2011). Another alternative could be the 5-(2-aminoethyl)-dithio-2-nitrobenzoate (ADNB), compound that is more stable in alkaline environment compared to DTNB and reacts similarly with thiols (Zhu et al. 2004).

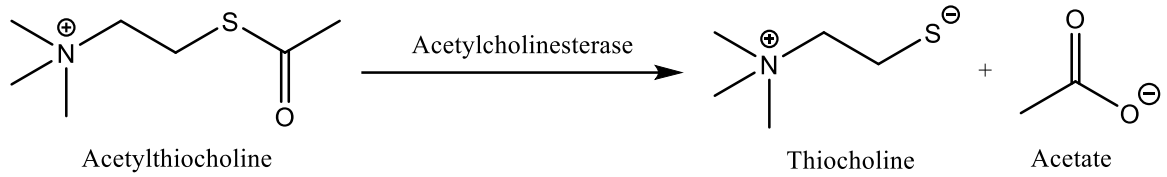


Figure 12. Hydrolysis of acetylthiocholine into thiocholine and acetate.

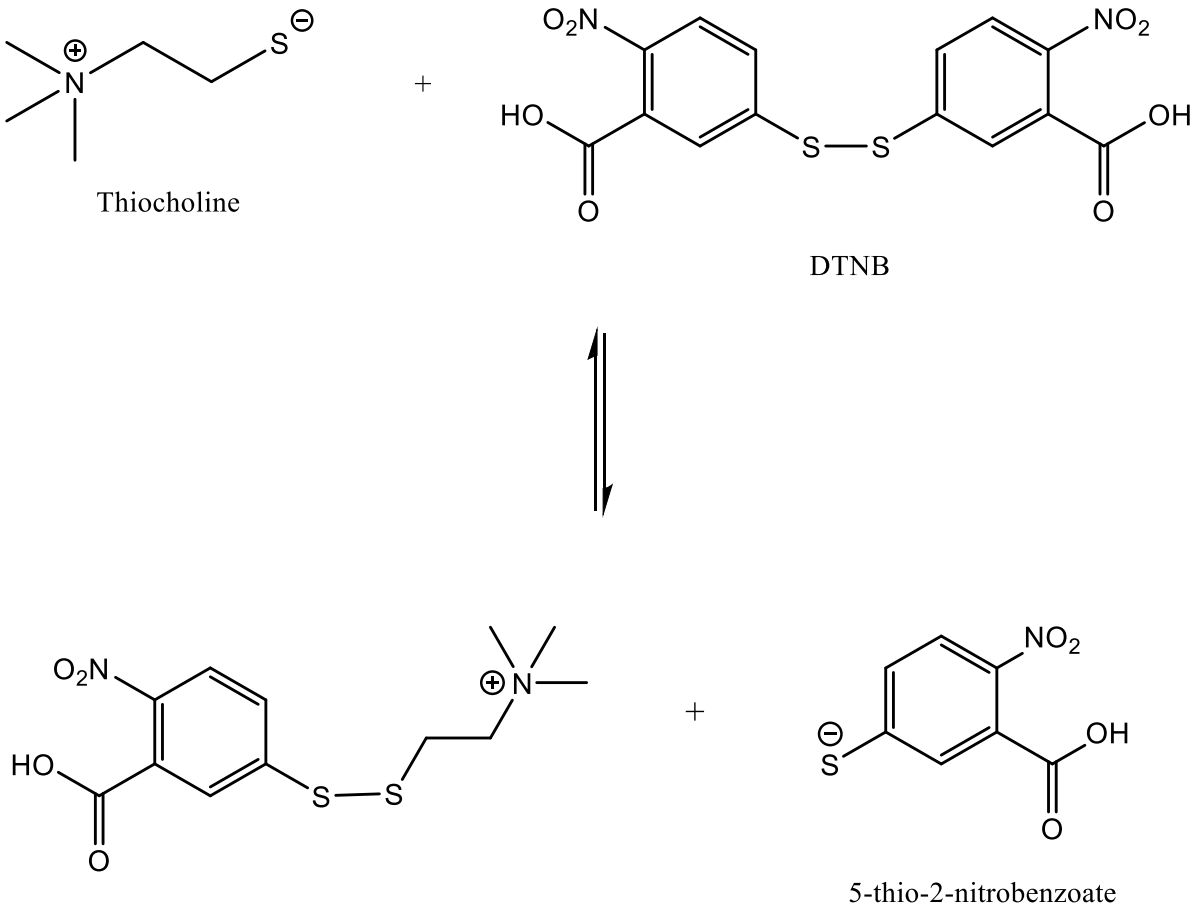


Figure 13. Cleavage of disulfide in DTNB molecule and subsequent reaction with thiocholine.

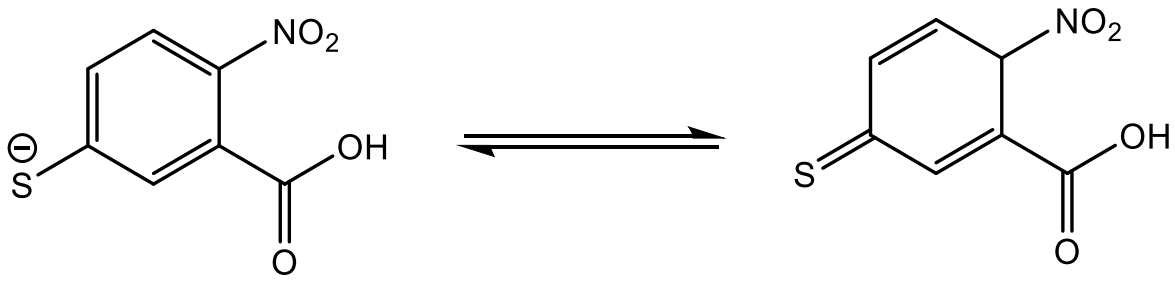


Figure 14. Resonance forms of TNB.[-]

4.2. Reagents and equipment

Reagents

Human acetylcholinesterase, prepared at the Department of Toxicology and Military Pharmacy

Mosquito acetylcholinesterase (Anopheles gambiae), prepared at the Department

Butyralcholinesterase, prepared at the Department

Acetylthiocholine, Sigma-Aldrich

Butyrylthiocholine, Sigma-Aldrich

Phosphate buffer, Sigma-Aldrich

Albumine, Sigma-Aldrich

5,5‘-dithiobis-2-nitrobenzoic acid (DTNB), Sigma-Aldrich

Dimethylsulfoxide (DMSO), Sigma-Aldrich

Distillled water

Equipment

Spectrophotometer Synergy 2 microplate reader, BioTek

Test tube shaker Lab Dancer, IKA

MS 3 Digital shaker for microplates, IKA

Magnetic stirrer Lab disc, IKA

Single channel micropipette Transferpette® S, adjustable, TreffLab/Brandtech

Multichannel micropipette Transferpette® S -12-12, adjustable, TreffLab

Multichannel micropipette Transferpette® S -8, adjustable, TreffLab

Pipet-Lite Adjustable Spacer LA8-50XLS pipette, Rainin

Autosampler BrandTech BRAND HandyStep Electronic Repeating Pipetter, BrandTech

96-Well Microplates, Nunc-Immuno

Polypropylene Centrifuge Tubes 10 mL, Corning™

Reagent reservoirs for multichannel micropipette, Brandtech

Eppendorf microcentrifuge tubes, 1.5 mL with lid, BRAND®

Eppendorf microcentrifuge tubes, 2 mL with lid, BRAND®

Analytical scales

Beakers

Plastic wash bottle

Pipette tips and LTS™ LiteTouch™ tips

Dispenser tips for HandyStep Electronic Repeating Pipetter

4.3. Preparation of solutions

Phosphate buffer 0,1 M, pH 7,4

Kalium dihydrogen phosphate KH_2PO_4 and sodium hydrogen phosphate Na_2HPO_4 were dissolved in distilled water in separate beakers. The solution of KH_2PO_4 was sequentially poured into a solution of Na_2HPO_4, until a pH of 7,4 was reached. The solution was stored in a plastic container with a lid in a refrigerator until further use.

Stock solution of DTNB

5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was dissolved with sustained stirring in phosphate buffer. Because of DNTBs photosensitivity, this solution had to be stored in plastic container impermeable to light during whole trial. The impact of light could induce cleavage of disulfide bridge and that would lead to the creation of TNB-. This process would result in a measurement deviation. Until next use, the solution was stored in a refrigerator.

Substrates

Stock solutions of substrates for enzymes of ATChI and BTChI were prepared by dissolution ATChI and BTChI in distilled water. Solutions were in volumes of 1,1 mL dispensed into Eppendorf microcentrifuge test tubes and stored in the refrigerator.

Enzymes

Human AChE (*h*AChE), mosquito AChE (*Ag*AChE), and BChE were prepared at the Department of Toxicology and Military Pharmacy in Hradec Králové. They were stored in Eppendorf microcentrifuge test tubes and stored in the refrigerator at -20°C.

Preparation of reagents for the assay

For the implementation of an assay, preparation of reagents, dilution series, and control of enzymatic activity had to be done in advance, just before the measurement.

Enzymes and their substrates were stored in the freezer until the next use. Thus, they had to be tempered to the required temperature before further manipulation.

After the complete thawing of ATChI and BTChI, 1 mL of each substrate was transferred into separate centrifuge tubes and 10 times diluted by adding 9 mL of distilled water. Subsequently, the solutions were shaken by Lab Dancer and poured into reservoirs for a multichannel micropipette.

2-3 mL of phosphate buffer was mixed with the same amount of distilled water and poured into reservoirs for a multichannel micropipette.

Due to its sensitivity, DTNB had to be kept in a reservoir impermeable to light during the entire measurement.

4.4. Preparation of dilution series

A set of twelve solutions with different concentrations were made for each tested AChEIs and their fragments.

Approximately 4 mg of the tested compound was weighed on analytical scales. Then the volume of solvent was calculated using the following equation/formula to acquire requested leaving concentration 10^{-2} M:

$$V = \frac{m}{Mr * c}$$

V - solvent volume (ml)
m - weight of tested compound (mg)
Mr - molar mass of tested compound
c - leaving concentration (10^{-2} M)

A weighed amount of sample was dissolved with a suitable solvent in a polypropylene centrifuge tube until complete dissolution using ultrasound. Dimethyl sulfoxide (DMSO) was used for dissolution of inhibitor, samples of tested fragments with sufficient water solubility were dissolved in distilled water. These solutions served as a basis for the preparation of the dilution series. From this point only distilled water was used as a thinner. Dilution series of samples were prepared in Eppendorf microcentrifuge tubes by the following procedure:

Solutions of the tested sample with decreasing concentrations by integer values were prepared by 10-fold dilution of previous concentration with distilled water

in Eppendorf 1,5 mL microcentrifuge tubes. Solutions with decreasing concentrations of intermediate half values were prepared from solution with closest higher integer value concentration.

Every test tube was shaken by Lab Dancer after every mixing and before every pipetting of solutions to ensure homogeneity.

4.5. Enzymatic activity control and regulation/adjustment

Before the assay, the control of activity for each enzyme was needed. 3 wells of 96-Well Microplate were used for each measurement (Figure 15.). As a substrate, ATChI was used for AChEs, BTChI for BChE.

Subsequently, the microplate was shaken with a digital shaker for microplates and inserted into a Synergy microplate reader, a device that performed the measurement. Required values of catalytic activity were in the range of 0,3 to 0,4. In case of deviation from the desired span, the adjustment of enzyme activity was done by dilution of *Ag*AChE with pure phosphate buffer, *h*AChE was diluted by a mixture of phosphate buffer and albumin. Solutions used for regulation of enzymatic activity were added with caution and continuous stirring, to prevent sudden changes that could damage AChEs. This process was repeated until the required enzyme activity was reached.

	1	2	3	4	5	6	7	8	9	10	11	12
A	hAChE	hAChE	hAChE	AgAChE	AgAChE	AgAChE	BChE	BChE	BChE			
B												
C												
D												
E												
F												
G												
H												

Figure 15. Microplate layout for enzymatic activity control.

4.6. Inhibitor activity determination

With acquired suitable enzyme activities, the measurement of inhibition rate for *h*AChE and *Ag*AChE by tested compounds and their fragments could be done. 96-Well Microplate was filled with reagents. Four rows of twelve wells were used for one trial of analysed structure. Every concentration was measured three times. Thus, three rows contained dilution series of inhibitor, fourth was control without tested compound. Firstly, the same amount of enzyme was pipetted into all selected wells. Then the phosphate buffer and DTNB were added. Solutions from the dilution series of inhibitor were added by Pipet-

Lite Adjustable Spacer LA8-50XLS pipette with decreasing concentration values from left to right side of the microplate, except wells with control. The microplate was subsequently shaken with digital shaker and inserted into a Synergy 2 microplate reader for inhibition for 5 minutes. At last, the substrate was added, quickly shaken, and put into a microplate reader for a 2-minute measurement.

As already stated, 4 rows (48 wells) were used for the determination of one inhibitor. Thus, the assay of two structures for one enzyme or one inhibitor for two enzymes at the same time was possible. In that case, only one series of control was needed (Figure 16.).

	1	2	3	4	5	6	7	8	9	10	11	12	
A	-4	-4,5	-5	-5,5	-6	-6,5	-7	-7,5	-8	-8,5	-9	-10	Inhibitor A
B	-4	-4,5	-5	-5,5	-6	-6,5	-7	-7,5	-8	-8,5	-9	-10	
C	-4	-4,5	-5	-5,5	-6	-6,5	-7	-7,5	-8	-8,5	-9	-10	
D	-4	-4,5	-5	-5,5	-6	-6,5	-7	-7,5	-8	-8,5	-9	-10	Inhibitor B
E	-4	-4,5	-5	-5,5	-6	-6,5	-7	-7,5	-8	-8,5	-9	-10	
F	-4	-4,5	-5	-5,5	-6	-6,5	-7	-7,5	-8	-8,5	-9	-10	
G													Control
H													

Figure 16. Microplate layout for measurement of AChE inhibition.

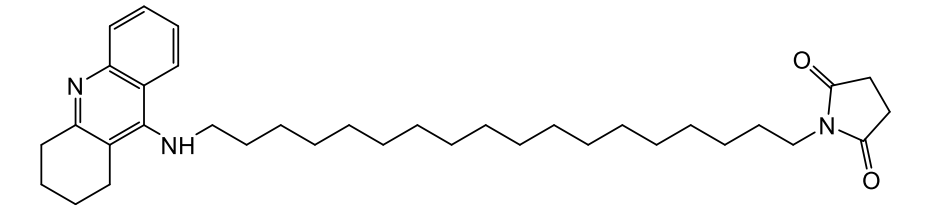
The inhibition rate of BChE by tested compounds was also assayed for selectivity comparison. But for each measurement, only six wells were used (Figure 17.). Three for inhibition and three for control. The same dosing procedure of solutions was applied as in AChE assay, but only one concentration value of inhibitor was measured (usually $10x10^{-4}$ for AChEI candidates and $10x10^{-3}$ for fragments).

	1	2	3	4	5	6	7
A	BChE inhibition	BChE inhibition	BChE inhibition	BChE control	BChE control	BChE control	
B							

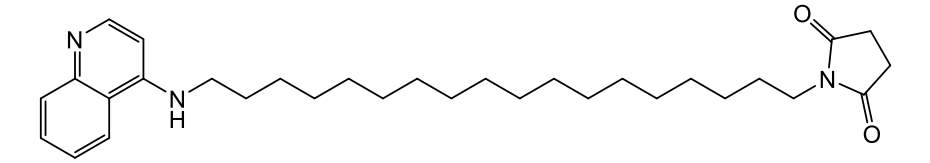
Figure 17. Microplate layout for measurement of BChE inhibition.

Measurement data were set to Delta A/min, results were saved to Microsoft Excel. Synergy 2 is a modular multimode reader. Ability to measure absorbance in ultraviolet to visible (UV-VIS) area was used for this diploma thesis.

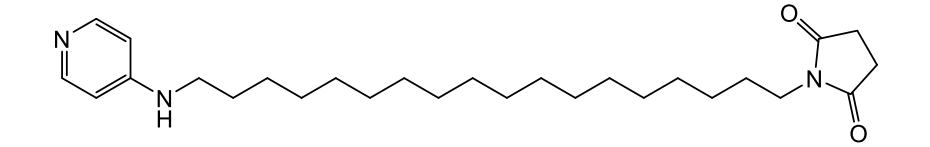
4.7. Tested structures



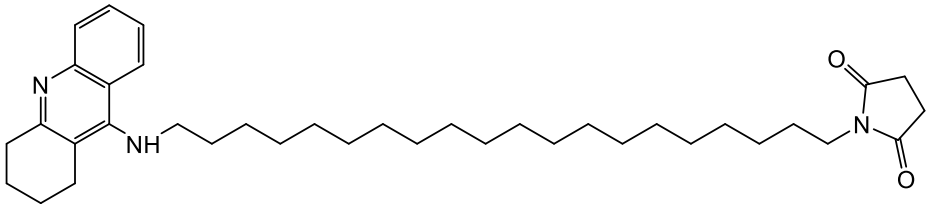
LG-488



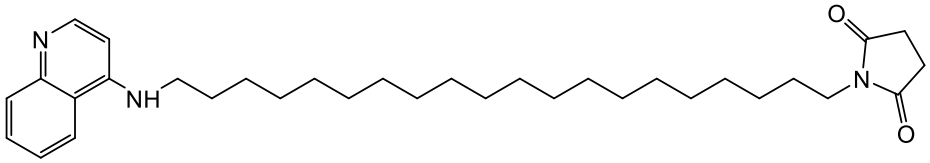
LG-505



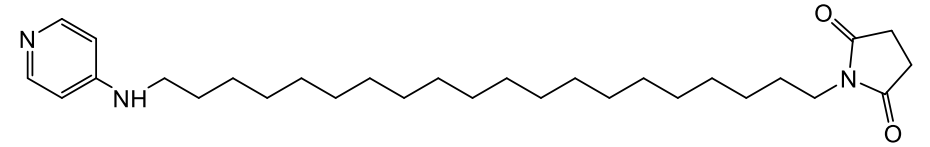
LG-495



LG-520

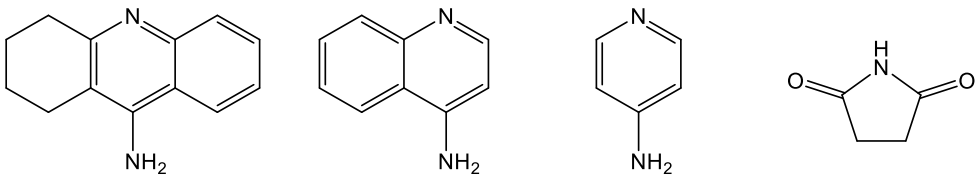


LG-525



LG-528

Figure 18. Structural formulas of novel compounds.



Tacrine | 4-aminoquinoline | 4-aminopyridine | Succinimide

Figure 19. Structural formulas of fragments.

5. RESULTS

Six succinimide derivatives as potential AChEIs were evaluated along with four fragments of their structures (tacrine, 4-aminoquinoline, 4-aminopyridine, and succinimide). Enzymatic activity was measured after the exposition to these compounds. The values of IC_{50} were determined for human and mosquito enzyme from the acquired data. Then the selectivity indexes (SI) were planned to be calculated, as a ratio of IC_{50} values for *h*AChE and *Ag*AChE. Additional measurement for activity against BChE was done.

$$SI = IC_{50}\ hAChE\ /\ IC_{50}\ AgAChE$$

Table 1. Novel compounds.

Name	*h*AChE (µM ± SEM)	*Ag*AChE (µM ± SEM)	BChE (µM ± SEM)
LG 488	0,569 ± 0,059	31 %	25 %
LG 495	35 %	18 %	12 %
LG 505	34 %	0 %	0 %
LG 520	4,88 ± 0,49	3 %	12 %
LG 525	31 %	0 %	2 %
LG 528	18 %	6 %	0 %

Note: SEM = standard error of mean

Table 2. Fragments.

Name	*h*AChE (µM ± SEM)	*Ag*AChE (µM ± SEM)	BChE (µM ± SEM)
Tacrine	0,291 ± 0,009	0,434 ± 0,015	0,073 ± 0,002
4-aminoquinoline	15,8 ± 0,6	40 %	23 %
4-aminopyridine	5 %	0 %	6 %
Succinimide (10^{-3} M)	0 %	3 %	0 %

Note: SEM = standard error of mean

IC_{50} could not be calculated when inhibition did not exceed 50 %, in this case percentage of inhibition at the highest tested concentration is stated instead.

5.1. Novel compounds

None of the six tested compounds showed the desired selectivity to mosquito enzyme. SI values could not be obtained since values of IC_{50} for both AChEs are needed

for its calculation. Measurement results are listed in Table 1. Structural formulas of compounds are listed in Figure 18.

Structures **LG488** and **LG520** possess **tacrine** (**THA**) skeleton connected to succinimide via aliphatic 18C and 20C long hydrocarbon chain, respectively. From among all novel compounds only these two demonstrated maximal inhibitory concentration against any of the enzymes. **LG488** in submicromolar (like **THA**) and **LG520** in micromolar range against *h*AChE. **LG488** inhibited *Ag*AChE and BChE the most from all the new substances, but this inhibition did not exceed 50 %.

Structures **LG505** and **LG525** possess **4-aminoquinoline** skeleton connected to succinimide via aliphatic 18C and 20C long hydrocarbon tether, respectively. These compounds showed no affinity to *Ag*AChE nor BChE. Rate of *h*AChE inhibition was similar, under 40 % in both structures.

Structures **LG495** and **LG528** possess **4-aminopyridine** skeleton tethered to succinimide via aliphatic 18C and 20C long hydrocarbon chain, respectively. **LG495** showed faint inhibition of each enzyme. **LG528** lowered *h*AChE activity the least of all novel compounds.

Structures with shorter tether length (18C) demonstrated slightly higher activity.

All tested compounds failed as insecticides since none was able to reach maximal inhibitory concentration in *Ag*AChE. Moreover, each substance had higher affinity to *h*AChE than *Ag*AChE.

5.2. Fragments

Four fragments of tested compounds were evaluated with intent to find relations between structure and activity. Measurement results are listed in Table 2. Structural formulas of fragments are listed in Figure 19.

THA and **4-aminoquinoline** demonstrated inhibitory activity towards each enzyme**. 4-aminoquinoline** reached IC_{50} in micromolar range for *h*AChE but inhibition of *Ag*AChE did not exceed 40%. Even in the highest concentrations, **4-aminopyridine** and **succinimide** had no impact on enzyme function. Results from measurement with 10^{-3} M concentration for **succnimide** are used due to inability of inhibition in lower concentrations and higher solubility.

As expected, **THA** showed IC_{50} in submicromolar range for *h*AChE, *Ag*AChE, and BChE. This indicates that **THA** fragment is responsible for ability of **LG488** and **LG520** to inhibit cholinesterases slightly more than other analysed compounds.

6. DISCUSSION

A large part of arthropod species is harmless and important for the ecosystem and humans. Pollinators (honeybees) help plants to reproduce, predators (spiders, mantises) regulate the numbers of other species, others accelerate the decomposition of cadavers, thus maintain ecological balance. Honey, bee wax and silk are examples of natural products gained from insects (Getanjaly et al. 2015).

On the other hand, some arthropods in large numbers cause enormous damage to the crops and disbalances in the environment (Culliney 2014). Moreover, insects make everyday life uncomfortable by biting and invading households and are vectors for multiple infectious diseases transmissible to humans and animals. Among vector-borne diseases belong yellow fever, Lyme disease, zika, malaria, dengue, chikungunya, lymphatic filariasis, Japanese encephalitis, and many more. Most of these infections are lethal (Engdahl 2017). Millions of people suffer from malaria every year (WHO 2019). Current chemoprophylaxis and pharmacotherapy are often ineffective. New drugs and vaccines are in development with the hope of finding a successful way to treat this infection (Ashley et al. 2018).

Regulation of malaria-vectors was proved to be an effective strategy to lower the number of infections. There are multiple methods of vector control, and usage of insecticides proved to be quite efficient. Unfortunately, excessive contact with these substances has a negative impact on human health, beneficial animals, and the ecosystem (Oberemok et al. 2015, Grieneisen and Zhang 2018, Pennetier et al. 2008). From among countless groups of chemicals, WHO recommends only four classes for usage in households (Najera and Zaim 2001).

Aside from their toxicity, there is another phenomenon occurring more frequently. After exposure to insecticide, insects adapt and develop various forms of resistance. Individuals can survive doses that would be otherwise lethal. Moreover, this tolerance can be passed on to the offspring. Thus, making insecticides counterproductive because they still can affect non-target species. There is an urgent need for new pesticides with sufficient safety and efficacy against insects (Oberemok et al. 2015, Grieneisen and Zhang 2018, Pennetier et al. 2008).

Organophosphates and carbamates are acetylcholinesterase targeting insecticides. Their mode of action is irreversible (OP) or reversible (CA) inhibition of AChE by blockage of serine residue inside of the CAS (Najera and Zaim 2001, Paudyal 2008). Thus, although these insecticides are very efficient, they are not species selective, because this serine is present in AChEs of both mammals and insects. Cysteine positioned near the entrance to the enzyme gorge is a new target of novel insecticides. This residue is insect-specific. Hypothetically, insecticides that target this amino acid could be selective to insects, effective against OP resistant strains, and had low toxicity to humans (Dou et al. 2013).

The focus of this work was *in vitro* evaluation IC_{50} of succinimide derivates as potential AChE inhibitors. Ideally, these values would be in the nanomolar range and selective to *Ag*AChE. Unfortunately, none of the analysed compounds met the conditions for the determination of IC_{50}. Without these values, the determination of selectivity between *h*AChE and *Ag*AChE was not possible.

An additional goal of this paper was an effort to find the relations between the structure and effect of these potential cholinergic inhibitors. Therefore, additional measurements of their fragments (THA, 4-aminoquinoline, 4-aminopyridine, and succinimide) were performed. Possible structure-activity relationship is discussed in the following paragraphs.

Tacrine is a known AChEI once used for the treatment of AD, now obsolete due to the adverse effects, especially hepatotoxicity (Wu et al. 2017, Svobodova et al. 2019). It has no significant selectivity between *h*AChE and *Ag*AChE, thus non modified monomer cannot be used for vector control. Based on that, prior to the measurements we assumed that THA derivatives, **LG488** and **LG520,** will exhibit inhibition towards AChE. THA results of IC_{50}, 0,291 ± 0,009 µM for *h*AChE and 0,434 ± 0,015 µM for *Ag*AChE confirm non-selectivity of this compound. **LG488** and **LG520** reached IC_{50} only in *h*AChE in concentrations 0,569 ± 0,059 µM and 4,88 ± 0,49 µM respectively, but inhibition of other two enzymes did not exceed 31% in either of the structures. Concentration needed to reach IC_{50} is two times higher in **LG488** than THA indicates that modifications in THA structure alter its inhibitory activity, in this case cause decline. None of potential AChEIs was able to inhibit *Ag*AChE more efficiently than *h*AChE. According to these results, THA seems to be better insecticidal candidate than any of the tested molecules.

THA was the only compound that reached IC_{50} value for BChE specifically in concentration 0,073 ± 0,002 µM. This outcome is consistent with the results of Svobodová et al. (2019) and Ahmed et al. (2006) who measured IC_{50} of 0,080 µM and 0,0256 µM respectively. Moreover, as in this work, both studies confirmed THAs higher affinity to BChE when compared to *h*AChE. Other compounds were not able to exceed 25 % inhibition of this enzyme.

Tacrine-squaramide compounds displayed significant inhibition of *h*AChE and *h*BChE (Svobodová et al. 2019). Wu et al. 2017 listed THA derivatives as potential medicaments for AD with cholinesterase inhibitory activity in humans. However, none of these compounds contained 4-aminopyridine, 4-aminoquinoline, nor succinimide moiety in their structures. Bis-tacrines with the tether length of 2 to 12 methylene units showed lower efficiency against mosquito AChEs (*An. gambiae*, *Culex pipiens*, *Aedes aegypti*) than *h*AChE (Anderson er al. 2009). Although these compounds were able to inhibit AChE of the insect species *Drosophila melanogaster* and *Blattella germanica* in micromolar to nanomolar concentrations, they were proven non-lethal because of their inability to cross the blood-brain barrier (Mutunga et al. 2013). These findings all together with the results of this work could indicate that THA derivatives are unfit as insecticides. Nevertheless, alterations in the structure remarkably alter the selectivity and potency.

Notable changes in inhibitory activity can be acquired through the substitution. Incorporation of functional groups (for example chlorine, methoxy) can cause the difference by a whole range (Wu et al. 2018, Svobodová et al. 2019). The potency of insecticide can be directly proportional to increasing or decreasing tether length but, in some cases, there is no direct dependence. Distinct structure and length of spacer affect not only efficacy but also selectivity even between related species (Anderson et al. 2009, Pang et al. 2009, Mutunga et al. 2013, Carlier et al. 2017). This suggests that the proper modifications of both acridine moiety of THA and side chain could lead to the synthesis of compounds with the desirable efficacy and selectivity toward insect AChE.

4-aminopyridine and succinimide fragments displayed no actual activity. In contrary to this, the linkage of these two completely ineffective compounds led to the creation of **LG495** and **LG528**, which showed slight inhibition. Hypothetically this can be due to the volumes of these compounds, correct alignment of molecules could cause obstruction of the gorge opening and thus restraint substrate to reach CAS. Moreover,

LG495 showed two times higher activity against *h*AChE than **LG528** what supports the fact that the tether length affects inhibitory properties.

4-aminoquinoline reached IC_{50} only in *h*AChE and none of its derivatives (**LG505** and **LG525**) was able to inhibit any of enzymes more than 35 %. Compounds with aminoquinoline moiety in their structure are studied as potential drugs for AD treatment (Chen et al. 2016, Wu et al. 2018). Based on our results, structures tested in this study are potential candidates for drugs to treat AD due to higher selectivity to *h*AChE. Likewise, it would be interesting to investigate selectivity towards *Ag*AChE of compounds mentioned in Chen et al. (2016) and Wu et al. (2018).

Aromatic rings of THA, 4-aminoquinoline, and 4-aminopyridine in the tested compounds are engaging via π-π or cation-π interactions with the indole ring of Trp 86 (*h*AChE) and Trp 87 (*Ag*AChE) located in the anionic subsite of CAS. As already been mentioned, the succinimide part was supposed to interact with the free cysteine residue in PAS of AgAChE, but the tested structures had a lower affinity toward this enzyme. This means that these novel inhibitors do not bind to Cys 286 of *Ag*AChE. Dou et al. 2013 came to the same conclusion. On the other hand, maleimide derivatives interact with Cys 286 through a carbon-sulfur bond. These compounds were able of conjugation with the insect-specific cysteine residue resulting in the selective irreversible inhibition of *Ag*AChE. The only difference between succinimide and maleimide is a double bond (Dou et al. 2013). This confirms the claim that even a slight alteration of structure can have a large impact on compound properties. *In silico* studies, for example, computer modelling or X-ray crystallography could shed light on the behaviour of succinimide part in the AChE gorge.

Structures evaluated in this study seem to be unfit for vector control. Their preference of *h*AChE to the *Ag*AChE points to the potential as cholinergic drugs in the therapy of humans. Nevertheless, structure modifications could result in the development of effective and safe insecticides. Such an adjustment could be the substitution of succinimide by maleimide. It leads to the increase of selectivity and affinity of substance towards anopheline AChE. Maleimide derivates should be subjected to further research since they possess the potential of insecticides with minimal effect in humans.

There is an urgent need to find new chemicals that would resemble safe, effective, and insect selective vector control. This could be done by targeting Cys 286, Arg 339, and their equivalents in other species, or both. Even though evaluated novel compounds failed as insecticides, the information obtained from this thesis will contribute to further research. The right alteration of existing insecticides may lead to the formulation of tools able to successfully tackle pests without posing a threat to humans.

7. CONCLUSION

The main purpose of this study was to explore if newly prepared succinimide derivatives can selectively inhibit *Ag*AChE *in vitro*. Their IC_{50} for *Ag*AChE and *h*AChE was measured.

Measurements showed that neither of the tested compounds has sufficient affinity towards mosquito enzyme. Only structures LG488 and LG520 reached values to determine IC_{50} in *h*AChE. IC_{50} values were in nanomolar and micromolar range respectively. None of the tested compounds reached 50 % inhibition in *Ag*ChE. This indicates their higher selectivity to human enzyme.

Based on these results we conclude that novel compounds failed as insecticides against mosquito *An. gambiae*, thus they cannot be used in vector control. Nevertheless, further modifications could lead to development of successful *Ag*AChE selective inhibitors.

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