Design and Synthesis of a Small Set of Thiourea-based Compounds as Inhibitors of AChE1 from Mosquitoes

Dariush Nikjoo
Abstract

Insect pests cause negative effects on human health and economy of both poor and
developing countries. Some problems like disease, crop-destroying and residential
insect pests, are serious and it is necessary to design and develop new and
environmentally safe insecticides to deal with the problems. Among vectors that
transmit disease, mosquitoes are a group of the vectors that cause disease like
malaria and yellow fever and result in serious suffering in the world.
Recently a high throughput screening (HTS) campaign towards Acetylcholinesterase
(AChE1) of two diseases transmitting mosquitoes, namely Anopheles gambiae (Ag,
malaria) and Aedes aegypti (Aa, Dengue, Yellow and Zika fever and Chikungunya)
have been performed. Analysis of the HTS resulted in the appearance of new class
compounds with potential for selective action between human AChE and
Ag/AaAChE1. In this study, one of the identified compound classes of HTS results
with the promising selectivity profile was evaluated to discussing on the structural
properties of compounds. A small set of analogues were targeted for initial structure-
activity relationship (SAR) using partial least square projection to latent structures
(PLS). In continue, the targeted compounds have been successfully synthesized and
purified for biological assay experiment. Four compounds were successfully
synthesized and evaluated for inhibition of AChE (Aa, Ag and hu). The well-known
Ellman assay was used for analysis of inhibitory potency of synthesized compounds.
Two compounds among the synthesized compounds show inhibition capacity.
Popular Scientific Summary

Insect pests could have negative effects on human health and economy of both poor and developing countries. Insect pests can destroy agricultural crops, harvest food, plants and cause illness in animals and humans. Therefore, it is necessary to design and develop new and environmentally safe insecticides to deal with the problems. Among insects that transmit disease, mosquitoes are a group of the insects that cause disease like malaria and yellow fever and result in serious suffering in the world. Enzymes are vital for organisms and acetylcholinesterase (AChE) is an enzyme that acts in the synaptic cleft of mammals, birds, and insects. The enzyme AChE, hydrolyses the neurotransmitter acetylcholine (ACh) to acetic acid and choline. In Alzheimer's and Huntington's disease, low production of ACh causes disorder in the transmitting of nerve signals. Researchers try to design and development of drugs that inhibit the enzyme and prevent the hydrolysis of the ACh and therefore increase nerve signaling. However, high dose of drugs, consequently deactivation of AChE and increase in choline cause clamp in healthy body and also deactivation of AChE in insects by insecticides lead to paralysis and death. This is the key point in design of new insecticides.

In this thesis project, a set of 36 molecules have been investigated to design of potential inhibitors of mosquitoes AChE enzyme. Mentioned set of molecules were discovered previously by investigation of AChE enzyme of two mosquitoes, namely Anopheles gambiae and Aedes aegypti which cause malaria and yellow fever respectively. The enzyme investigation has been done by a developed method and using professional instruments. A descriptor that explain different parts of molecules were made according to obtained 36 molecules and then computer software was used to analyze and prediction of activity of compounds. Four compounds were targeted according to obtained modeling results. Synthesis and purification of target compounds were the next step in this project. Furthermore, the affinity and binding to AChE evaluated by biological assay for synthesized compounds. Biological assay was done by using the prepared enzyme from the mosquitoes and synthesized molecules. Finally, the results were evaluated and compared to the results predicted by the model.

The importance of this work was the design and synthesis of new insecticides. This project was done using computer software's to classify and predict biological activity of molecules with certain error. Currently, computational methods are being used to find starting point of drug and insecticide design and development projects. According to our findings we can say molecules with non-aromatic amines as well as long aliphatic chain connecting to amine can be suitable molecules for discovering of new insecticides.
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<th>Definition</th>
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<tr>
<td>AC</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ACHE</td>
<td>Acetylcholinesterase</td>
</tr>
<tr>
<td>hACHE</td>
<td>Human Acetylcholinesterase</td>
</tr>
<tr>
<td>ATCI</td>
<td>Acetylthiocholine iodide</td>
</tr>
<tr>
<td>AcOH</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>Ag</td>
<td>Anopheles gambiae</td>
</tr>
<tr>
<td>Aa</td>
<td>Aedes aegypti</td>
</tr>
<tr>
<td>CAS</td>
<td>Catalytic anionic site</td>
</tr>
<tr>
<td>PAS</td>
<td>Peripheral anionic site</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMF</td>
<td>N, N'-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-Dithiobis-(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>HTS</td>
<td>High throughput screening</td>
</tr>
<tr>
<td>hu</td>
<td>Human</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>MeCN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>Mol.</td>
<td>Molecular</td>
</tr>
<tr>
<td>MWI</td>
<td>Microwave irradiation</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OAc</td>
<td>Acetate</td>
</tr>
<tr>
<td>Obsd</td>
<td>Observed</td>
</tr>
<tr>
<td>Ph</td>
<td>Phenyl</td>
</tr>
<tr>
<td>PLS</td>
<td>Partial least square projection to latent structures</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure–activity relationship</td>
</tr>
<tr>
<td>rt</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationship</td>
</tr>
<tr>
<td>Sat.</td>
<td>Saturated</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
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</table>
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1. Introduction

Many infections can be transmitted between humans or from animals to humans by vectors. During the blood meal of insects, disease-producing micro-organisms are ingested by the vectors from an infected host (human or animal) and later inject to a new host in the next blood meal. Some of the diseases that are caused by vectors are mosquito-borne diseases, tick-borne diseases, Rodent-borne diseases and others. Among the vectors mosquitoes are the best known disease vectors. Vector-borne diseases are caused by pathogens and parasites in human populations and infect more than one billion people and kill one million people in the world. Some of the diseases caused by vectors are Malaria, Dengue, Schistosomiasis, Leishmaniasis, Chagas disease, Yellow fever, Lymphatic filariasis and Onchocerciasis. Currently more than half of the world’s population estimated to be at risk of vector borne diseases. The poor parts of society and least-developed countries and vulnerable sub-populations, such as children under age of five and pregnant women are most affected.

The most effective ways to prevent transmission of the diseases such as malaria are the use of insecticide treated nets and indoor residual spraying. Bed-nets should be checked regularly for holes and changed every 2–3 years. Also, at least 80% of the houses in the targeted area need to spray and it is effective for just 3-6 month and it should be renewed after this period. Furthermore, researchers should start with synthesis and development of new effective, safe and sustainable insecticides that is motivated by increasing resistance development and generic toxicity of currently used insecticides.

The enzyme acetylcholinesterase (AChE) hydrolyses the neurotransmitter acetylcholine (ACh) to acetic acid and choline in the synaptic cleft of mammals, birds, and insects. However, deactivation of AChE in insects by insecticides lead to paralysis and death. Among the insects, mosquitoes show extensive and strong resistance to the insecticide. According to the literature except mammals and some flies, most disease-transmitting, crop-damaging and residential insects have two AChE genes. One is AChE1 that is responsible for insecticide resistance in mosquitoes and AChE2 that is orthologous to the human AChE.

1.1 Aim of the diploma work

Recently high throughput screening (HTS) have been accomplished towards AChE1 of two diseases transmitting mosquitoes, namely Anopheles gambiae (Ag, malaria) and Aedes aegypti (Aa, Dengue, Yellow and Zika fever and Chikungunya). Analysis of the HTS resulted in the identification of new classes of compounds with potential for selective action between hAChE and Ag/AaAChE1. These new classes are starting points to develop and synthesize of new and selective inhibitors. Indeed, the aim of this study was to chemically modify a hit compound identified in a high throughput screening campaign (Figure 1).

![Hit compound discovered in a HTS campaign](image)

Modifying the hit and choosing the target compounds was according to the initial structure-activity relationship (SAR) and a partial least square (PLS) model. Synthesis and purification of target compound was the next step in this project. The affinity and binding to AChE can be evaluated by changing of substituents on the amine group and the number of carbons in the aliphatic chain of the hit. The goal is building the initial SAR by data from HTS and synthesizing the new class of compound with potential to AChE1 inhibition. Finally, the results will be evaluated according to SAR point of view and compared with results predicted by the model.
2. Background

2.1 What is a vector and why is it important

Vectors are living organisms with potential to transmit infectious diseases between humans or from animals to humans. These diseases affect human life and especially people with poverty living condition like lack of access to adequate housing, safe drinking water and sanitation. Also, these diseases cause difficulties for people to work which result in further poverty and hindering economic development. Some of the vectors that transmit diseases are mosquitoes, ticks, rodents and other pests like bed bugs, body and head lice, africanized honey bees, red imported fire ants, and yellow jackets triatoma. Figure 2 shows some of these vectors and corresponding disease as examples.

Figure 2. (a) Tick cause tick fever, (b) Rat cause rat bite fever, (c) Anopheles gambiae mosquito cause malaria, (d) Aedes aegypti mosquito cause yellow fever

Some diseases that are caused by vectors are very dangerous and they don’t have effective remedy, like West Nile Virus and Dengue fever and for these disease vectors control is the only way to protect populations.

Vector control is any process to prevent the vectors that transmit disease pathogens. The most frequent type of vector control is mosquito control using a variety of strategies. However, for vector-borne diseases with treatments the high cost of treatment is a huge barrier to economy of the developing world population. Malaria is a treatable disease, however; malaria has the greatest impact on human health from vectors.

African malaria mosquitoes (Anopheles gambiae) that transmit malaria, sicken approximately 207 million (with an uncertainty range of 135 million to 287 million) and kills nearly 627 000 people (with an uncertainty range of 473 000 to 789 000) in 2012. Children were the most important target for malaria victims and every 60 seconds a child died from malaria disease. In countries where malaria is well controlled, the countries lose 1.3% annual economic income due to the disease. Furthermore, both prevention with vector control and treatment are necessary to protect populations.

2.2 Vector born diseases

Table 1 shows some diseases that are caused by vectors. The most common vectors that lead to disease are mosquitoes.

Table 1. Some of vectors along with their transmitting diseases

<table>
<thead>
<tr>
<th>Vectors</th>
<th>Type</th>
<th>Disease</th>
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<tbody>
<tr>
<td>Mosquitoes</td>
<td>Anopheles</td>
<td>Malaria, lymphatic filariasis</td>
</tr>
<tr>
<td></td>
<td>Aedes aegypti</td>
<td>Dengue, yellow fever, chikungunya, Zika virus</td>
</tr>
<tr>
<td></td>
<td>Aedes albopictus</td>
<td>Chikungunya, dengue, West Nile virus</td>
</tr>
<tr>
<td></td>
<td>Culex quinquefasciatus</td>
<td>Lymphatic filariasis</td>
</tr>
<tr>
<td></td>
<td>Haemagogus</td>
<td>Yellow fever</td>
</tr>
<tr>
<td>Sandflies</td>
<td></td>
<td>Leishmaniasis</td>
</tr>
<tr>
<td>Triatomine bugs</td>
<td></td>
<td>Chagas disease</td>
</tr>
<tr>
<td>Ticks</td>
<td>Crimean-Congo haemorrhagic fever, tick-borne encephalitis, typhus, Lyme disease</td>
<td></td>
</tr>
<tr>
<td>Fleas</td>
<td></td>
<td>Plague, Murine typhus</td>
</tr>
<tr>
<td>Flies</td>
<td></td>
<td>Human African trypanosomiasis, onchocerciasis</td>
</tr>
</tbody>
</table>
2.2.1 Malaria
Malaria is a type of disease that causes fever, chills and it is a flu-like illness. Early diagnosis is very important in the treatment of malaria and delay in diagnosing can lead to disease progression or sometimes it may lead to death. After a mosquito bit, it takes seven days that the symptoms of malaria appear. There is not any commercial vaccine for malaria. However, a potential vaccine against plasmodium falciparum is currently in clinical trials in seven African countries. Plasmodium known as malaria parasite and plasmodium falciparum is one of the species of plasmodium that transmit by female anopheles mosquito and cause malaria in humans.\(^1\)

2.2.2 Dengue
Dengue fever is a serious, flu-like illness. High fever, intense headaches, joint and muscle pains, nausea, vomiting and rash are some of dengue symptoms. Dengue is not serious and rarely lead to death, but in some cases which appear with symptoms such as low temperature, bleeding gums, rapid breathing, intense abdominal pains, and blood in vomit, can be fatal.\(^1\)

2.2.3 Chikungunya
Fever and intense joint pain are symptoms of chikungunya that can last for weeks. Muscle pain, nausea, fatigue, headache, and rash are also other symptoms of chikungunya. Almost all patients are fully recovered, but in some cases joint pain may continue for several months, or even years. Often symptoms are moderate and the infection may be not identified, or be incorrectly diagnosed in areas where infection occurs. Some cases of heart, neurological and eye complications have been reported, as well as gastrointestinal complaints. Serious complications are not common, but in older people, the disease can lead to death.\(^1\)

2.2.4 Yellow fever
Yellow fever was one of the most dangerous, fatal diseases before the development of an effective vaccine. After infection, symptoms of the disease usually appear in 3–6 days. The first step is characterized by fever, loss of appetite, headache, shivers, muscle pain, nausea and vomiting. After 3–4 days, most patients become better and symptoms disappear. But, almost 15% of patients enter a toxic phase; fever returns and the patient develop jaundice and sometimes bleeding. The most important medication against yellow fever is vaccination and usually, a single dose of the vaccine is enough to life-long immunity.\(^1\)

2.3 Regulation of Acetyl choline

Figure 3 shows natural regulation of the amount of ACh reaching the cholinergic receptors. In case of nerve agents, permanent inhibitions of AChE by covalent bonding to the catalytic serine residue of AChE lead to increasing in amount of ACh in the synaptic cleft. In healthy adults this leads to cramps because of continuous signalling to the muscles.\(^9,10\) In Alzheimer disease, patients have a low production of the ACh and that can be treated by a reversible AChE inhibitors like Donepezil. It is the active substance in the drug Aricept.\(^11\)
2.4 Acetylcholinesterase biological action

The enzyme acetylcholinesterase (AChE) hydrolyses the neurotransmitter acetylcholine (ACh) into acetic acid and choline in the synaptic cleft (Figure 4 and 5b).

\[
\begin{align*}
\text{Acetylcholine} & \xrightarrow{\text{AChE}} \text{Acetic Acid} + \text{Choline} \\
\end{align*}
\]

AChE contains an active site, shaped as a 20 Å deep gorge and two sub sites. These sub sites are the peripheral anionic site (PAS) and the catalytic anionic site (CAS). PAS is located at the entrance of the active site and CAS is located at the bottom of the gorge. These sites are targets for natural or artificial inhibitors (Figure 5a)

Aromatic residues at the PAS interact with cationic part of the ligands and the ligands travels down the gorge and bind the anionic CAS. Ligands that bind to the AChE are generally basic and contain positively charged nitrogen. Also, some ligands contain aromatic systems that make interaction with PAS.
Organophosphates and Carbamates are the two well-known insecticides that effect on the AChE enzyme. Organophosphates inhibit AChE, causing ACh to increase at cholinergic synapses and this lead to neuromuscular paralysis and other effects. This can occurred through the stable bond between organophosphate and serine amino acid. Carbamate insecticides have similar action but shorter duration of action and are less toxic.  

### 2.5 Acetylcholinesterase in insects

Acetylcholinesterase in insects have two genes. One is AChE1 which has the main synaptic activity and is responsible for insecticide resistance in mosquitoes and AChE2 that is orthologous to the human AChE.  

Figure 6 (a) shows the active-site gorge of AChE1 showing the locations of the peripheral and catalytic sites and conjugation of chlorpyrifos, that is a crystalline organophosphate insecticide, with the catalytic serine residue.  

Also Figure 6 (b) shows the structural difference between mammals and insect AChE. The green structure is homology model of AChE1 from Anopheles gambiae and the white structure is the AChE from human. As can be seen in the figure the difference is in the peripheral site and both mammalian and insect enzymes have serine amino acid in the CAS site. In mammalian enzyme the amino acid in the PAS is phenylalanine with phenyl group and for insect enzyme is cysteine with thiol group.

![Figure 6.](image)

**Figure 6.** (a) The active-site of AChE1 and the locations of the CAS (1) and PAS (2) in conjugation of chlorpyrifos with the catalytic serine reside (Adopted from ref.2) (b) Structural difference of the active site between mammalian and insect AChE, green structure is homology model of AChE1 (Ag) and the white structure is the hAChE.  

Targeting serine residue in mammalian enzyme by insecticides causes toxicity. The, insect-specific cysteine residue at the opening of the AChE1 active site is a promising target site for developing new insecticides with reduced off-target toxicity and low trend for insect resistance. Scientists follow the hypothesis that this residue is a novel target site for insecticide development and move to design of cysteine target insecticides.  

### 2.6 Structure analysis relationship (SAR)

Structure-activity relationship (SAR) is one of the important parts of medicinal chemistry which tries to correlate the chemical structure to the observed activity. Medicinal chemists use the SAR to pick up new chemical groups into the biomedical compound that lead to modification in their biological activities. Compounds are selected for syntheses that maximize the presence of functional groups or properties that improve the activity of the compound. The basic assumption is that similar molecules have similar activities. This assumption result in a problem and that is how to define a small difference on a molecular level like reaction ability, biotransformation ability, solubility, target activity, and so on. Indeed in practice, the SAR refers to the fact that all similar molecules have not similar activities.  

### 2.7 Partial least squares (PLS)

Partial least squares projections to latent structures (PLS), is a method related to principal component analysis, that the information is divided by two blocks of variables, X and Y, which are connected between them. PLS is used in different areas like quantitative structure-activity relationship (QSAR)
modeling, multivariate calibration, process monitoring and optimization. In PLS each observation is represented by two points, one in the X-space and one in the Y-space and the objective of the test is to establish a relationship between the position of the observations in the predictor space (X) and their positions in the response space (Y). \(^{15}\)

PLS model work based on two equations that try to establish a relationship between X and Y and for prediction of Y from X. These objectives are shown in these two equations:

\[
\begin{align*}
X &= 1x' + TP' + E \\
Y &= 1y' + UC' + F
\end{align*}
\]

Where \(x'\) and \(y'\) represent the variable averages. \(T\) and \(U\) are the score matrices where the information related to the observations is stored in \(P'\) and \(C'\). The variation in the data that cannot be modeled is stored into the residual matrices, \(E\) and \(F\).

\(R^2_X\) (cum) describes how much of the variation in the data matrix \(X\) is described by the model and \(Q^2_X\) (cum) describes the model's ability to predict the variation in the data set indeed it is the internal prediction. \(^{15}\)

2.8 Biological assay

Biological evaluation of AChE can be used for recognition of activity after exposure to organophosphorus or carbamate pesticides and nerve agents. Also biological assay can be used for determination of treatment effectiveness of Alzheimer’s disease inhibitors or new potential pesticides. In this study we have elucidated the potency and selectivity of our four compounds by measuring the change in enzymatic activity in the presence of inhibitor at various concentrations. Experimental protocol for AChE activity assay has been proposed and the most common assay is based on Ellman's method. \(^{16}\) This method is based on using an alternative substrate, acetylthiocholine (ATCI), and the reagent 5, 5'-dithio-bis-2-nitrobenzoic acid (DTNB) and the reaction results in production of 5-thio-2-nitrobenzoate with yellow color. \(^{17}\) The change in absorbance over time in this assay is direct proportional to the enzymatic activity.

![Figure 7. The reaction of the enzymatic degradation of ATCI and the formation of the yellow product after reaction with DTNB.](image)

The change in color is because of transferring electrons to the sulfur atom. The method was developed by Ellman\(^ {16}\) and coworkers in the early 1960s and researchers modified the method over the years. \(^{17}\)

3. Experimental

3.1 Methods and Materials

For synthesis of compounds microwave method was used by a monomode reactor (Smith Creator, Biotage AB) in Teflon capped 2-5 ml Smith TM process vials with stirring. Microwave reactions were carried out with dry solvent under anhydrous conditions. \(^{18}\) For evaluation of reactions Thin layer chromatography (TLC) were carry out using the Silica gel 60 F254 (Merck) plates and visualizing with UV light, ninhydrin (0.75 g ninhydrin, 250 ml 95% EtOH, 2.5 mL glacial acetic acid) for diagnosis of amines. The reactions were checked using LC-MS (Waters Micromass ZQ Electrospray (ES+) and (ES-) instrument with column XTerra® MS C18 5 µM 4.6×50 mm H₂O/acetonitrile eluent system). Samples
were solved in MeOH and filtered prior to LC-MS. For structural characterization of the compounds, \(^1\)H and \(^{13}\)C NMR spectra were used on a Bruker DRX-400 instrument in CDCl\(_3\) [residual CHCl\(_3\) (δ \(H\) 7.26 ppm, δ \(C\) 77.16 ppm)] or (CD\(_3\))\(_2\)SO [residual (CH\(_3\))\(_2\)SO (δ \(H\) 2.50 ppm, δ \(C\) 39.52 ppm)]. NMR spectra Carbon and proton resonances were determined by COSY and HETCOR experiments. IR spectra were recorded on an ATI Mattson Genesis Series FTIR spectrometer. Products were purified with crystallization method with different type of solvent systems depending on the compound.

### 3.2 PLS Analysis

#### 3.2.1 PLS projections

According to HTS results that recently have been performed \(^5\) 34 compounds have been obtained. The HTS results lead to a hit compound (Figure 1) that has been used for the initial SAR and PLS study. For PLS analysis, 25 descriptors has been made according to the difference in amine group, number of aliphatic chains and also different in the benzene group for definition of matrices \(X\). Table 2 shows the different descriptors along with abbreviations for them.

<table>
<thead>
<tr>
<th>NO</th>
<th>Abbreviation</th>
<th>explanation</th>
<th>NO</th>
<th>Abbreviation</th>
<th>explanation</th>
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<tr>
<td>1</td>
<td>Meto_o</td>
<td>Metoxy in orto position</td>
<td>14</td>
<td>Nitro_p</td>
<td>Nitro in para position</td>
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<td>2</td>
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<td>Metoxy in meta position</td>
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<td>4</td>
<td>F_o</td>
<td>Fluor in orto position</td>
<td>17</td>
<td>NO_c_3</td>
<td>Number of carbon 3</td>
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<td>5</td>
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<td>Fluor in meta position</td>
<td>18</td>
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<tr>
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<td>Ring2</td>
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</tr>
<tr>
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<tr>
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<tr>
<td>9</td>
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</tr>
<tr>
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<td>Ring6</td>
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<td>Ring8</td>
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<td>SO2_NH2_p</td>
<td>Para Sulfonamide</td>
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</table>

As shown in the Table 2, 25 descriptors has been made according to change in different part of the hit compound. As the \(Y\) matrices the inhibition percentages at 50 micro molar have been used from the HTS results. The PLS model was calculated after importing the descriptors in Simca\(^{19}\) software. Figure 8 shows the PLS projection.\(^{15}\)

![Figure 8. Illustration of matrices in PLS projection](image)

PLS model has been calculated with one principal component and cross-validation method has been used for determination of principle component. R\(^2\)X, R\(^2\)Y and Q\(^2\) values were 0.095, 0.67 and 0.11 respectively.
3.3 General procedure for synthesis of DN0001, 1-(3-chloro-phenyl)-3-(2-piperidin-1-yl-ethyl)-thiourea
1-Chloro-3-isothiocyanato-benzene (77 μL, 0.5 mmol) was added in dry DCM (5 mL) in the vial of microwave under stirring. Then, 2-piperidine-1-yl-ethylamine (80 μL, 0.5 mmol) was added to the solution drop by drop. The reaction has been done by microwave synthesis at 70 °C for 15 minutes. After the reaction time has ended EtOAc (10 ml) was added to the reaction flask, and the organic phase was washed once with NaHCO₃ (aq, satd). The organic phase was washed with brine solution to remove extra water. All organic phases were dried over anhydrous Na₂SO₄ and removed under reduced pressure. The products were purified using crystallization with (heptane/DCM 5:1) which leads to a white solid product (191 mg, 96 % yield). IR (neat) 3338, 3152, 3002, 2935, 2851, 2770, 1581, 1516, 1428, 1282, 1246, 1187, 1113, 1074, 870, 791, 767, 695, 635cm⁻¹. ¹H NMR (400 MHz, DMSO) δ 1.44 (m, 2H), 1.55 (m, 4H), 2.41 (s, 4H), 2.54 (m, 2H), 3.60 (s, 2H), 7.18 (m, 1H), 7.36 (m, 2H), 7.75 (s, 1H), 9.80 (s, 1H), 9.87 (s, 1H); ³¹C NMR (100 MHz, DMSO) δ 179.8, 140.9, 132.7, 130.2, 123.4, 121.7, 120.6, 56.4, 53.8, 41.1, 25.5, 24.0. LC/MS m/z calculated C₁₄H₂₀ClN₃S: 297.84 and C₁₄H₂₀ClN₃S: 298.1(M+H)⁺

3.4 General procedure for synthesis of DN0002, 1-(3-Chloro-phenyl)-3-(2-morpholin-4-1-ethyl)-thiourea
1-Chloro-3-isothiocyanato-benzene (77 μL, 0.5 mmol) was added in dry DCM (5 mL) in the vial of microwave under stirring. Then, 2-ethyl-1-yl-ethylamine (77 μL, 0.6 mmol) was added to the solution drop by drop. The reaction has been done by microwave synthesis at 70 °C for 15 minutes. After the reaction time has ended EtOAc (10 ml) was added to the reaction flask, and the organic phase was washed once with NaHCO₃ (aq, satd). The organic phase was washed with brine solution to remove extra water. All organic phases were dried over anhydrous Na₂SO₄ and removed under reduced pressure. The products were purified using crystallization with (heptane/DCM 5:1) which leads to a white solid product (173.1 mg, 98 % yield). IR (neat) 3168, 3006, 2940, 2864, 2795, 1582.01, 1517, 1431, 1268, 1245, 1114, 1071, 1023, 954, 870, 855, 796, 771, 695cm⁻¹. ¹H NMR (400 MHz, DMSO) δ 2.49 (m, 2H), 2.58 (m, 4H), 3.67 (m, 6H), 7.22 (m, 1H), 7.40 (m, 2H), 7.79 (s, 1H), 7.89 (s, 1H), 9.90 (s, 1H); ³¹C NMR (100 MHz, DMSO) δ 179.9, 140.9, 132.7, 130.2, 123.4, 121.7, 120.7, 66.1, 56.3, 53.1, 40.6. LC/MS m/z calculated C₁₄H₂₀ClN₃S: 299.84 and C₁₄H₂₀ClN₃S: 300.1(M+H)⁺

3.5 General procedure for synthesis of DN0003, 1-(3-Chloro-phenyl)-3-(2-imethylamino-thyl)-thiourea
1-Chloro-3-isothiocyanato-benzene (77 μL, 0.5 mmol) was added in dry DCM (5 mL) in the vial of microwave under stirring. Then N,N-dimethyl-ethylenediamine (64 μL, 0.5 mmol) was added to the solution drop by drop. The reaction has been done by microwave synthesis at 70 °C for 15 minutes. After the reaction time has ended EtOAc (10 ml) was added to the reaction flask, and the organic phase was washed once with NaHCO₃ (aq, satd). The organic phase was washed with brine solution to remove extra water again. All organic phases were dried over anhydrous Na₂SO₄ and removed under reduced pressure. The products were purified using crystallization with (heptane/DCM 5:1) which leads to a white solid product (130 mg, 86 % yield). IR (neat) 3172, 3006, 2943, 2819, 2770, 1589, 1517, 1473, 1281, 1074, 1038, 875, 769, 693cm⁻¹. ¹H NMR (400 MHz, DMSO) δ 2.25 (s, 6H), 2.56 (t, 2H), 3.60 (s, 2H), 7.17 (m, 1H), 7.38 (m, 2H), 7.86 (s, 2H), 9.90 (s, 1H); ³¹C NMR (100 MHz, DMSO) δ 180.0, 141.2, 132.6, 130.0, 123.2, 121.5, 120.5, 57.0, 44.9, 41.6. LC/MS m/z calculated C₁₄H₁₈ClN₃OS: 257.78 and C₁₄H₁₈ClN₃S: 258.02(M+H)⁺

3.6 General procedure for synthesis of DN0004, 1-(3-Chloro-phenyl)-3-(3-dimethylamino-propyl)-thiourea
1-Chloro-3-isothiocyanato-benzene (77 μL, 0.5 mmol) was added in dry DCM (5 mL) in the vial of microwave under stirring. Then 3-(dimethylamino)-1-propylamine (74 μL, 0.5 mmol) was added to the solution drop by drop. The reaction has been done by microwave synthesis at 70 °C for 15 minutes. After the reaction time has ended, EtOAc (10 ml) was added to the reaction flask, and the organic phase was washed once with NaHCO₃ (aq, satd). The organic phase was washed with brine solution to remove extra water again. All organic phases were dried over anhydrous Na₂SO₄ and removed under reduced pressure. The products were purified using crystallization with (heptane/DCM 5:1) which leads to a white solid product (158.4 mg, 98 % yield). IR (neat) 2941, 2855, 2821, 2774, 1591, 1515.60, 1460, 1291, 1230, 1191, 1155, 1095, 1074, 1036, 995, 930, 879, 775, 680, 613cm⁻¹. ¹H NMR (400 MHz, DMSO) δ 1.65 (m, 2H), 2.08 (s, 6H), 2.25 (t, 2H), 3.48 (m, 2H), 7.14 (m, 1H), 7.31 (m, 2H), 7.62 (s, 1H), 8.16 (s, 1H), 8.07 (s, 1H), 7.31 (m, 2H).
3.7 IC50 value determination of compounds
The inhibition ability of the synthesized compounds was evaluated by determination of the IC50 (the concentration needed of the compound to inhibit half the enzymatic activity) values for three target AChE proteins. The target proteins were hAChE and AChE1 of two mosquitoes (see above). Protein preparations have been done at FOI Umea, by Cecilia Engdahl (Umea university), and all measurements were carried out on secreted non-purified enzymes in serum-free cell culture media.

Ellman’s essay was used for evaluation of inhibition potency. Briefly, stock solutions (100 mM) of four synthesized ligands were prepared in DMSO as solvent. The ligands were prepared and tested in different concentrations as: 1, 0.2, 0.1, 0.01, 0.001, 0.0001, 0.00001, and 0.000001 mM in the assay. Assay has been done in total volume of 200 μl in wells as follow protocol:
- 160 μL Phosphate buffer (pH 7.4, 0.1 M)
- 10 μL of AChE enzyme in Phosphate buffer (pH 7, 0.1 M)
- 10 μL of DTNB (4 mM) in Phosphate buffer (pH 7, 0.1 M)
- 10 μL of the inhibitor, 1-0.000001 mM, (In blanks only DMSO was added)
- 10 μL of ATCI (20 mM) in water

ATCI, DTNB and the enzyme was kept on ice during the experiment. The ATCI was added last for all repeats and as quick as possible since the reactions start immediately. The optical density was measured at 405 nm in 11 s intervals for two minutes using the plate reader Spectra Max 340. The IC50 values were then calculated from the dose-response curves. In some cases that IC50 was not concluded from the curves, the inhibition was just stated as inhibition at specific concentrations. All compounds were tested in triplicates on at two separate occasions.

4. Results and Discussion

4.1 Finding building blocks

A descriptor was generated according to changes in different parts of hit compounds from HTS results. Descriptor was used in initial SAR which resulted in choosing the new chemical groups and new biomedical compounds as targeted compounds. Retro synthesis of targeted compounds was carried out in order to see which building blocks are needed to form the compounds. When the required reactions and building blocks were identified, Sigma-Aldrich and Sci-Finder® was checked for suitable starting materials and synthetic methods.

4.2 PLS analysis of the building blocks

In order to choose different chemical groups for the new analogues a PLS study was performed. A descriptor was made according to the difference in amine group, number of carbon in aliphatic chain and also different in the benzene. The used properties for descriptors and obtained results for the PLS study have been shown in Figure 9.
The total number of properties for making descriptor was 25. The descriptor matrix and inhibition percentages at 50 micro molar, were imported to SimcaP® and a PLS model was created with one component. The Q2(Y(cum)) value was 0.11, meaning that the model can predict 11% of the variation through cross validation. The R2(Y(cum)) value was 0.67, meaning that the model could describe 67% of the variation in the data. Regarding to the R2 and Q2 values, the model is not a good model and could not predict well. However, the aim of PLS study is just making a starting point for synthesis of new compounds in regard to HTS results with certain uncertainty. Furthermore, the inhibition values for HTS results are values with high percentage of errors, since they are not IC50 values and tested at one concentration and time with no replicates. To make a satisfied model with good certainty, a collection with large number of compounds and convincing IC50 values are necessary. As can be concluded from the model, the properties in descriptor that are above zero line have a positive effect on the inhibitory potential and the properties under the zero line have a negative effect on the inhibitory potential. For an instant the ring 1, 2, 5 and 4 have the positive effect and rings 7, 8, 3, and 6 have a negative effect on the inhibitory potential of the compound. Also, according to model aliphatic chain with two carbons has a positive effect and chains with three carbons have a negative effect on the inhibitory potential. Finally, according to the model phenyl group with meta-chloro substituent has a positive effect on the inhibition potential for an instance. According to the obtained results, four compounds were targeted for synthesis and investigation of inhibition potential.

4.3 Synthesis of analogues

4.3.1 General procedure for synthesis of target compounds

Organic synthesis is another important part of research in medicinal chemistry. Among the methods of organic synthesis, microwave irradiation became popular in the last decade as a rapid and efficient synthesis method because of selective absorption of microwave energy by polar molecules. In microwave synthesis, irradiation lead to a rapid rise in the temperature of reaction and the process is not limited by the disadvantages of conventional methods. In conventional heating, heat pass through the walls of reaction vessel and reach to solvent and reactants and reactants are slowly activated by external heat source. General procedure for synthesis of target compounds along with reactants, reaction condition and yields is shown in Figure 10. The synthesis was performed in one step and microwave synthesis method was used. Starting with 1-chloro-3-isothiocyanato-benzene (1) and starting materials (2a, 2b, 2c and 2d) and using microwave synthesis (70 °C for 15 minutes) resulted in determined products successfully (3a, 3b, 3c and 3d).
Figure 10. The synthesis of N-substituted thioureas through primary amine–isothiocyanate coupling.

The target products purified by crystallization with (heptane/DCM 5:1) and led to a white solid pure product with Yields: 96%, 98%, 86% and 98% for target compounds 1 to 4 respectively.

4.4 Proposed mechanism

Figure 11 shows the possible mechanism of the N-substituted thioureas reaction between amine and isothiocyanate. The reaction starts with microwave irradiation and nucleophilic amine attacks to isothiocyanate. Subsequently, acidic hydrogen can be eliminated by the basic nitrogen that leads to target product.

Figure 11. The proposed reaction mechanism of N-substituted thioureas reaction.

4.5 Biological evaluation

Four compounds were successfully synthesized and evaluated for inhibition of AChE (Aa, Ag and hu). Their inhibitory potencies were tested at different concentrations using the Ellman assay, in order to establish the concentration which inhibited 50% of the enzyme activity (IC₅₀), and the percentage of inhibition value. There was a problem with preparation of the high concentrations in biological assay that may have contributed to the variation in the results. The first concentrations for the compound 1 (DN0001) and compound 2 (DN0002) were high and it seems the ligand did not completely dissolve. Tests were performed in triplicates and two different occasions. Figure 12 shows the relative activities of enzyme versus log concentration for compounds as inhibitors. Relative activities are in relation to inhibition of enzymes and when the concentration of ligand is low the relative activity of enzyme is in maximum values. In contrast, if we move to high concentrations activity of enzyme decrease.
Among the synthesized compound, DN0004 was the best inhibitor of the compounds and compounds DN0001 and DN0002 showed low inhibition activities. According to the figures DN0001 and DN0002, relative activity goes to constant values with increasing in the concentration. In these cases increasing in the concentration dose not hinders the enzyme activities and we do not have any inhibition. In case of DN0003 and DN0004 increasing in the concentration cause decrease in the activities that means ligands successfully hinder the enzyme. Furthermore, according to Figure 12, for all compounds, there is not any selectivity in the inhibitors among the different kind of enzymes (Aa, Ag and hu). The lack of full dose-response curves makes IC50 values uncertain. Thus we prefer to report just graphs for potential inhibition.

4.6 Structure-Activity Relationship

Two of the analogues (DN0003 and DN0004) showed good inhibition of AChE1 at high concentration. They both have the dimethylamine group and the difference between compounds is in the number of carbons in the aliphatic chain attached to the amine. Furthermore, both of the ligands have a meta-chloro benzene group that it maybe could have potential interaction with the PAS of the enzyme. However, DN0004 has a slightly better inhibition potency that we should look for the reason in the interactions in the catalytic site of the enzyme. This might be due to the flexibility of the dimethylamine group in the DN0004 because of the high number of carbons. Flexibility may help amine chain to make interaction and better ionic interaction with the CAS of AChE1.

DN0001 and DN0002 did not show good inhibition. Structural difference is in the amine groups. It seems cyclic amines did not have good interaction with the active site of the enzymes in our study. Also, the results for Dn0001 were different from the prediction of the PLS model. According to the result for
PLS model, piperidine ring should have good interaction with the enzymes. Also the high number of carbon resulted in good inhibition that it is different from the PLS predictions.

As a conclusion, when we go to the nonaromatic amine and the high number of carbons in the lead compound, inhibition increase. However, the number of compounds in the experiment is very limited and for getting the reliable rules the SAR collection compounds should be extended.

5. Conclusions

In this work, we used a simple initial SAR for studying of the collection of compounds obtained from HTS. PLS model was used to identify the target compounds by making a descriptor according to results of HTS. Overall four compounds synthesized and purified. Convenient and environmentally friendly microwave synthesis was used for production of the compounds. Several methods have been used for characterization of the synthesized compounds. Consequently, compounds were evaluated for inhibition of three proteins of AChE (Aa, Ag and hu). Their inhibitory potency was tested using Ellman assay. Finally, the obtained results analyzed, compared with the predicted results and possible interactions were discussed. Two compounds show good inhibition but there is no selectivity between enzymes.

6. Acknowledgement

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7. Appendix

$^1$H NMR (400 MHz, DMSO) and $^{13}$C NMR (100 MHz, DMSO) spectra for the DN0001.
$^1\text{H NMR}$ (400 MHz, DMSO) and $^{13}\text{C NMR}$ (100 MHz, DMSO) spectra for the DN0002.
$^1$H NMR (400 MHz, DMSO) and $^{13}$C NMR (100 MHz, DMSO) spectra for the DN0003.
\(^1\)H NMR (400 MHz, DMSO) and \(^{13}\)C NMR (100 MHz, DMSO) spectra for the DN0004.
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